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(54) **Expression vectors for enhanced production of polypeptides, plasmids containing the vectors, hosts containing the plasmids, products manufactured thereby and related methods.**

(57) An improved vector upon introduction into a suitable bacterial host containing the thermolabile repressor CI renders the host cell capable, upon increasing the temperature of the host cell to a temperature at which the repressor is destroyed, of effecting expression of a desired gene inserted into the vector and production of polypeptide encoded by the gene. The vector is a double-stranded DNA molecule which includes in 5' to 3' order the following: a DNA sequence which contains the promoter and operator $P_{L}O_{L}$ from lambda bacteriophage; the N utilization site for binding antiterminator N protein produced by the host cell; a DNA sequence which contains a ribosomal binding site for rendering the mRNA of the desired gene capable of binding to ribosomes within the host cell; and ATG initiation codon or a DNA sequence which is converted into an ATG initiation codon upon insertion of the desired gene into the vector; a restriction enzyme site for inserting the desired gene into the vector in phase with the ATG initiation codon; and additionally a DNA sequence which contains an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell and a DNA sequence which contains a gene associated with a selectable or identifiable trait which is manifested when the vector is present in the host cell.

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EXPRESSION VECTORS FOR ENHANCED PRODUCTION OF POLYPEPTIDES, PLASMIDS CONTAINING THE VECTORS, HOSTS CONTAINING THE PLASMIDS, PRODUCTS MANUFACTURED THEREBY AND RELATED METHODS

BACKGROUND OF THE INVENTION

One aspect of genetic engineering involves the insertion of foreign DNA sequences derived from eukaryotic sources into Escherichia coli or other microorganisms. A further refinement of genetic engineering concerns inducing the resulting microorganism to produce polypeptides encoded by the foreign DNA. Production of polypeptides can be considered a two-step process, with each step including numerous substeps. The two steps are transcription and translation. To produce a polypeptide efficiently and in quantity both steps of the process must be efficient. Transcription is the production of mRNA from the gene (DNA). Translation is the production of polypeptide from the mRNA.

A critical substep of the transcription process is initiation, that is, the binding of RNA polymerase to a promoter-operator region. The sequence of deoxyribonucleotide bases which make up the promoter region may vary and thereby effect the relative efficiency of the promoter. The efficiency depends on the affinity of the RNA polymerase for the promoter.

The efficiency of translation is affected by the stability of the mRNA. Increased stability of the mRNA permits improved translation. Although the exact determinants of mRNA stability are not precisely known, it is known that

mRNA secondary structure as determined by the sequence of its bases has a role in stability.

The initial substep of translation involves binding of the ribosome to a base sequence on the mRNA known as the Shine-Dalgarno sequence or the ribosomal binding site (RBS). The synthesis of polypeptides begins when the ribosome migrates along the mRNA to the AUG start codon for translation. Generally these codons are found approximately 10 bases "downstream" from the Shine-Dalgarno site. Factors which increase the efficiency of translation include those which enhance binding of the ribosomes to the Shine-Dalgarno site. It has been shown that the secondary structure of the mRNA in the region of the Shine-Dalgarno sequence and the AUG codon and the distance between the Shine-Dalgarno sequence and the AUG codon each play a critical role in determining the efficiency of translation. Other factors which affect the efficiency of translation are premature termination and attenuation. Efficiency of translation can be improved by removing the attenuation sites.

A difficulty encountered in attempts to produce high amounts of eukaryotic polypeptides in bacterial cells involves the inability of cells producing large amounts of mRNA to grow efficiently. This difficulty can be eliminated by preventing transcription by a process known as repression. In repression genes are switched off due to the action of a protein inhibitor (repressor protein) which prevents transcription by binding to the operator region. After microorganisms have grown to desired cell densities, the repressed genes are activated by destruction of the repressor or by addition of molecules known as inducers which overcome the effect of the repressor.

Numerous reports may be found in the literature concerning the cloning of eucaryotic genes in plasmids containing the P_L promoter from λ bacteriophage. (Bernard, H.V. et al., Gene (1979) 5, 59; Derom, C. et al., Gene (1982) 17, 45; Gheysen, D. et al., Gene (1982) 17, 55; Hedgpeth, J. et al., Mol. Gen. Genet. (1978) 163, 197; Remaut, E. et al., (1981) Gene 15, 81; and Derynck, R., et al., Nature (1980) 287, 193. In addition, European Patent Application No. 041.767, published December 16, 1981 describes expression vectors containing the P_L promoter from λ bacteriophage. However, none of these references describe the use of the C_{II} ribosomal binding site.

The use of a vector containing the P_L promoter from λ bacteriophage and the C_{II} ribosomal binding site has been described. (Oppenheim, A.B. et al., J. Mol. Biol. (1982) 158, 327 and Shimatake, H. and Rosenberg, M., Nature (1981) 292, 128.) These publications describe the production of increased levels of C_{II} protein but do not involve or describe the production of eucaryotic proteins.

In 1982 Shatzman and Rosenberg presented a poster at the 14th Miami Winter Symposium (Shatzman, A.R. and Rosenberg, M., 14 Miami Winter Symposium, abstract p98 [1982]). This abstract provides a non-enabling disclosure of the use of a vector containing P_L from λ bacteriophage, Nut and the C_{II} ribosomal binding site to synthesize a "eucaryotic" polypeptide (SV40 small T antigen is actually not a eucaryotic polypeptide but a viral protein) in an amount greater than 5% of the cell protein in an unnamed bacterial host. The operator used is not defined. Neither an origin of replication nor a gene for a selectable phenotype is identified. This system with which the vector is used is described as including certain host lysogens into which the vector can be stably transformed. The present invention in

one embodiment, i.e., pMG100, may have certain similarities to this vector. However, it is not transformed into a host lysogen, but rather into suitable E. coli host strains which contain the thermolabile repressor C_I and the N gene but from which the rest of the lysogen has been removed. Moreover, it has been employed to produce bGH and hGH analogs in amounts in excess of 20% of total cell protein.

In addition, in other embodiments of this invention ribosomal binding sites which differ from C_{II} are employed. Also, in the presently most preferred vectors, pND5 and its derivatives, nonessential sequences have been removed to create a vector permitting polypeptide production in amounts which are more than 10% greater than those obtained with pMG100.

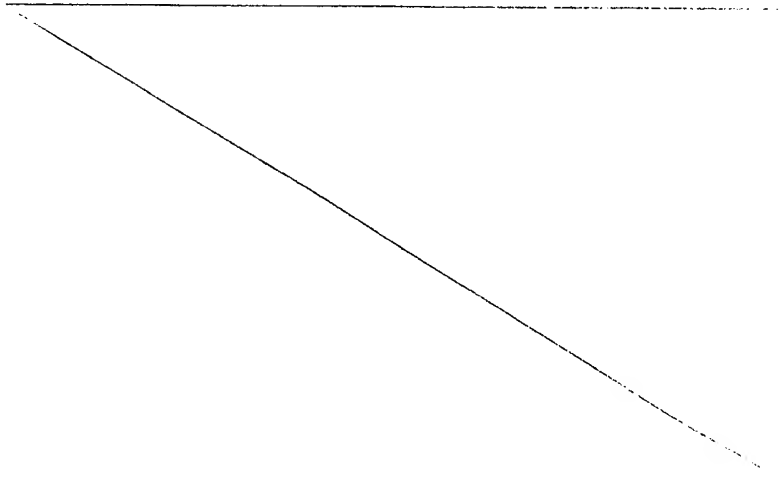
Recently, applicants have learned of the existence of a pending U.S. patent application in the name of M. Rosenberg filed under Serial No. 457,352 by the National Institutes of Health, Dept. of Health and Human Services, U.S.A. Portions of this application have been obtained from the National Technical Information Service, U.S. Dept. of Commerce. However, the claims are not available and are maintained in confidence. The available portions of the application have been reviewed. This disclosure is not enabling. It indicates that the host is important (p8, line 17) but fails to identify any suitable host. It further depends upon the use of a λ mutant which is not specified (p4, line 20). It indicates that the host contains lysogens (p8, line 18) unlike the present invention in which the host is not lysogenic. It mentions cloning and expression of a eucaryotic gene, monkey metallothionein gene, (p7, line 18) but does not provide details. It specifies that neither the sequence nor the position of any nucleotide in the C_{II}

ribosomal inding region has been altered. (p3, line 27) In the present invention such alteration is possible.

No disclosure is present in the art concerning: successful expression with a P_L-C_{II} containing vector system of bovine or human growth hormones; production of bGH or hGH analogs having biological activity; compositions containing such analogs or uses of them; or induction methods for achieving polypeptide production in amounts greater than 20% of the total protein produced by the host.

The only disclosure in the art concerning production of bGH analogs by hosts transformed with genetically engineered vectors involves the use of the Trp promoter to produce a bGH analog having the amino acid Met at the N-terminus of the phenylalanine form of natural bGH (Seeburg, P.H. et al., DNA (1983) 2, 37.

The only disclosure in the art concerning production of hGH analogs by hosts transformed with genetically engineered vectors involves the use of the Lac and Trps promoters to produce an analog of hGH having the amino acid Met at the N-terminus of the natural hGH. (Goedell, D.V. et al., Nature (1979) 281, 544)



SUMMARY OF THE INVENTION

This invention concerns an improved expression vector which upon introduction into a suitable bacterial host cell, namely, Escherichia coli, containing the thermolabile repressor C_I renders the host cell capable, upon increasing the temperature of the host cell to a temperature at which the repressor is destroyed, of effecting expression of a desired gene inserted into the vector and production of the polypeptide encoded by the gene comprising:

a double-stranded DNA molecule which includes in 5' to 3' order the following:

a DNA sequence which contains the promoter and operator P_LO_L from lambda bacteriophage;

the N utilization site for binding antiterminator N protein produced by the host cell;

a DNA sequence which contains a ribosomal binding site for rendering the mRNA of the desired gene capable of binding to ribosomes within the host cell;

an ATG initiation codon or a DNA sequence which is converted into an ATG initiation codon upon insertion of the desired gene into the vector; and

a restriction enzyme site for inserting the desired gene into the vector in phase with the ATG initiation codon;

and which additionally includes a DNA sequence which contains an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell and a DNA

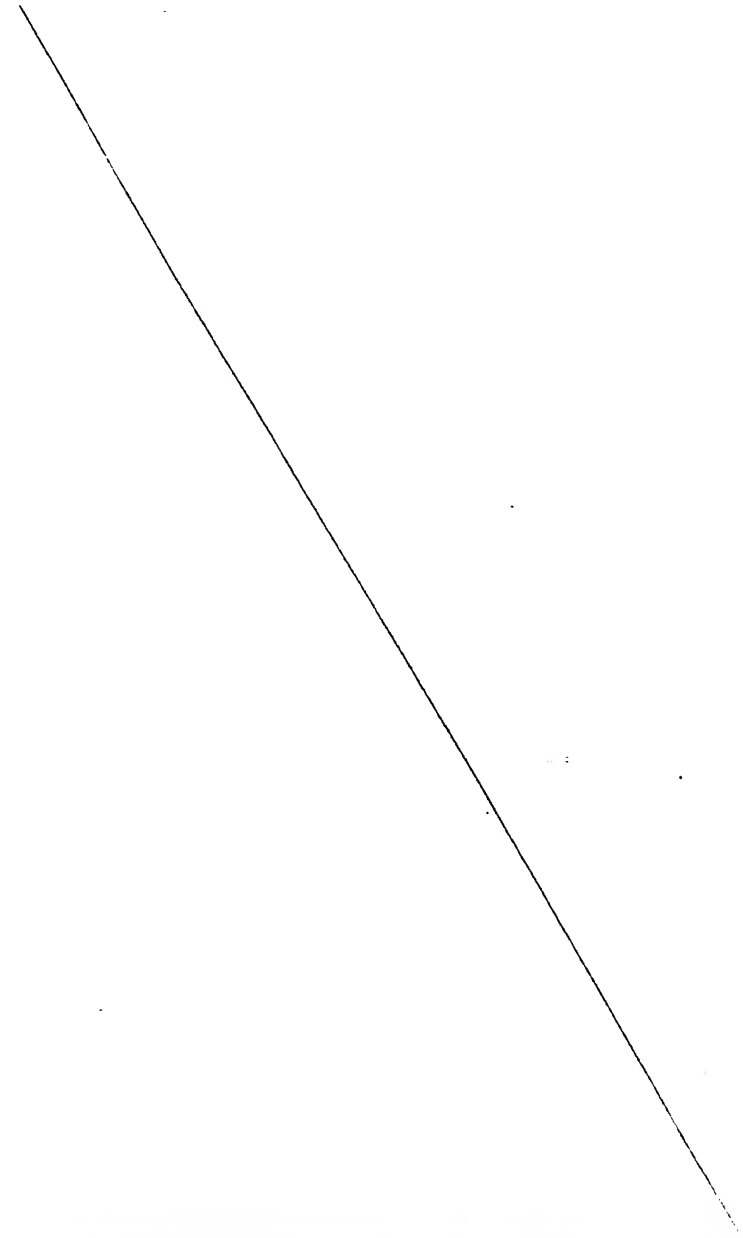
sequence which contains a gene associated with a selectable or identifiable phenotypic trait which is manifested when the vector is present in the host cell. Preferred vectors are pMG 100 and pND5.

Genes, i.e., cDNAs, encoding desired polypeptides such as growth hormones, e.g., bovine, porcine, chicken or human growth hormones, superoxide dismutase, apoprotein E, viral protein 1 of foot and mouth disease virus, protein A from S. aureus, interleukin III, an enzyme or analogs thereof may be inserted into the restriction enzyme site of the vector to create plasmids. The plasmids in turn can be introduced into suitable hosts where the genes can be expressed and the desired polypeptide produced. Preferred plasmids for bGH are pRec 2/3 and pRO11; and for hGH, pTV 18(1) and pTV 104(2). Suitable hosts include Escherichia coli A1637, A1645, A2602 and A1563; A1637 being presently preferred.

The resulting host vector systems can be employed to manufacture polypeptides. The host cells containing the plasmids are grown under suitable conditions permitting production of polypeptide and the resulting polypeptide is recovered. Presently preferred conditions involve growth at about 42°C for 10 to 30 minutes, particularly 15 minutes, followed by continued growth at about 37-39°C for sufficient time to make the total growth period about 60-90 minutes, particularly growth at 38-39 °C for about 75 minutes. Presently preferred growth media are lactalbumin hydrolysate with addition of glucose or brain heart infusion.

Using the host-vector systems, analogs of bGH and hGH have been prepared. These analogs may be incorporated into veterinary or pharmaceutical compositions, respectively.

The respective analogs directly, or in such compositions, may be used to stimulate milk or meat production in a bovine or to treat human growth hormone deficiency.



DESCRIPTION OF THE FIGURES

FIG. 1. Construction of pMG100 expression vector. This plasmid was built by inserting a fragment of λ phage DNA contained between restriction sites HaeIII (location 38150) and Sau3a (location 38362) into a pKC30 plasmid DNA cleaved with HpaI and BamHI. The HaeIII-Sau3a fragment carries nut_R, t_{R1}, cy^- and ribosomal binding site of C_{II} protein (C_{II}-RBS). Subcloning of the C_{II}-RBS containing DNA into pKC30 creates pMG100 which contains a unique BamHI restriction site right after the ATG initiation codon of C_{II}-RBS and an NdeI restriction site within the ATG triplet (bottom inset). Numbers in parentheses denote location of restriction sites on the λ phage DNA.

FIG. 2. Construction of pRec 2/3 plasmid. A bGH cDNA containing plasmid, D_4, was digested with HaeII. A resulting 1600 bp large fragment was purified and subjected to digestion at 37°C for 5 minutes with 5 units of S1 exonuclease. A synthetic EcoRI linker with the sequence:

GGAATTCC
CCTTAAGG

was attached by ligation. The product was cleaved with EcoRI and inserted into pBR322 which had been cleaved with EcoRI. A clone, pALR1, was isolated which upon cleavage with EcoRI released a 1200 bp fragment with the sequence:

AATTCCCA....
GGGT....

at the 5' end. Formation of this sequence demonstrates that pALR1 contains an EcoRI restriction site which includes the TTC codon for residue number 1 (phenylalanine) of authentic bGH. pALR1 was subjected to a partial cleavage with PstI. The digest was ligated with HindIII linkers and cleaved

with EcoR1 and HindIII. The fragment containing bGH cDNA was isolated and subcloned into pBR322 between EcoR1 and HindIII restriction sites to give pAL500. The subcloned bGH cDNA fragment was then excised from pAL500 with EcoR1 and HindIII, "filled in" with DNA polymerase "Klenow" fragment and inserted into the pMG100 expression vector (FIG. 1)opened at the BamH1 site and also "filled in" as above. The resulting vector pREC 2/2, expresses a modified bGH which is altered at its amino terminus as follows:

$$MetAspGlnPhe^{1}Pro^{2}.....bGH$$

The plasmid pREC 2/2 was digested with PstI and the fragment containing the P_L promoter and the 5' end of the bGH gene (designated fragment A) was isolated. This fragment was ligated to a PstI fragment from pAL 500 (designated fragment B). The then resulting vector, pRec 2/3, expresses a modified bGH which is altered at its amino terminus as follows:

$$MetAspGlnPhe^{1}Pro^{2}.....bGH$$

FIG. 3. Construction of expression vectors pND5, pND55 and pROl1. A plasmid pOG7 (A. Oppenheim, S. Gottesman and M. Gottesman, J. Mol. Biol. (1982) 158, 327) was cleaved with NdeI. The ends of the large fragment carrying the P_L promoter nut_L, t_R and C_{II}-RBS were ligated to give the pND5 expression vector. This pND5 vector DNA is opened with NdeI. Insertion of that NdeI fragment from pRec 2/3 (FIG. 2) which contains bGH cDNA results in a plasmid pROl1 which appears to be a better expressor of the modified bGH described in FIG. 2 than pRec 2/3. Insertion of synthetic linkers with the sequence:

TATGAGCTCA

ACTCGAGTAT

into pOG7 cleaved with NdeI results in an expression vector pND55 which contains a unique SacI restriction site in

front of ATG. When pND55 is cleaved with SacI and treated with DNA polymerase "Klenow" fragment an ATG initiation codon results which follows the P_L promoter and C_{II}-RBS. This vector is suitable for expression of a wide variety of eukaryotic genes lacking an ATG initiation codon.

FIG. 4 Construction of pTV 18(1) and pTV 104(2). A plasmid, pTVHGH was prepared by cloning cDNA encoding hGH into the HindIII site of pBR 322 using standard methods. Meth. Enzymol. (1979) 68, 75. This plasmid was digested with HindIII. The resulting 800 base pair fragment was purified and further digested with FnuDII and "filled in" with DNA polymerase "Klenow" fragment. This treatment removes codons for the first 16 amino acids of hGH. The resulting DNA fragment is ligated with a synthetic linker which restores the codons for the sequence of hGH from Met^{14} and regenerates an NdeI restriction site in front of the ATG codon for Met^{14}. After treatment with NdeI this semi-synthetic DNA was inserted into the pND5 vector opened with NdeI. The resulting plasmid pTV 18(1) expresses hGH under control of the P_L promoter. This hGH is an analog missing the first 13 amino acid residues and having at its N-terminus Met^{14}.

Plasmid pTV 18(1) was partially digested with NdeI and ligated with a synthetic linker which contains the codons for amino acids 1-13 of hGH:

TATGTTCCCAACCATTCCATTATCCCGTCTGTTCGACAACGC
ACAAGGGTTGGTAAGGTAATAGGGCAGACAAGCTGTTGCGAT.

The linker is also complementary to the NdeI site on pTV 18(1) and positions the complete hGH gene in phase with the ATG initiation codon of the pND5 expression vector (FIG.

3). Thus, the resulting plasmid, pTV 104(2), expresses native hGH with an extra methionine at the N-terminus.

FIG. 5 shows the vector pAL Trp 46 which contains the Trp promoter and the first seven amino acids of the Trp E gene transcriptionally fused to the β-galactosidase gene.

FIGS. 6, 7 and 8 show a series of expression vectors (Tac) containing a part of the Trp promoter and Lac operator followed by restriction sites for insertion of a desired gene and expression of bGH under the control of Tac promoter.

FIGS. 9 and 10 show expression vectors containing bGH cDNA under the control of the histidine promoter.

FIG. 11 shows insertion of the bGH gene into an expression vector under the control of the Lac promoter.

FIG. 12 shows expression of bGH gene under control of Omp F promoter.

FIG. 13. Construction of Met^4-bGH analog pAL401 and expression vectors pND6 and pND11 with altered restriction sites:

pAL401 which expresses a modified form of bGH which is lacking the first three amino acids at the amino terminus of the bGH (Met^4 bGH) was constructed by triple ligation

of the following:

a) a bGH DNA fragment of 623 base pairs with PvuII and HindIII excised from pAL500

b) a linker formed by synthesizing two DNA strands which after purification were annealed to form:

CCATATGTCCTTGTCCGGCCTGTTTGCCAACGCTGTGCT

GCGACACGAGGCCCGAGTCGTGGACGTGGTCGACG

which was "filled in" with DNA polymerase "Klenow" fragment and then cleaved with NdeI and PvuII to prepare a 58 base pair fragment which was recovered and purified.

c) pND11 which was prepared as follows. An expression vector pOG7 was altered by elimination of HindIII and one of the NdeI sites (distant from the ATG initiator codon) to obtain pND6. Then HindIII linkers were introduced into a SalI site to give pND11.

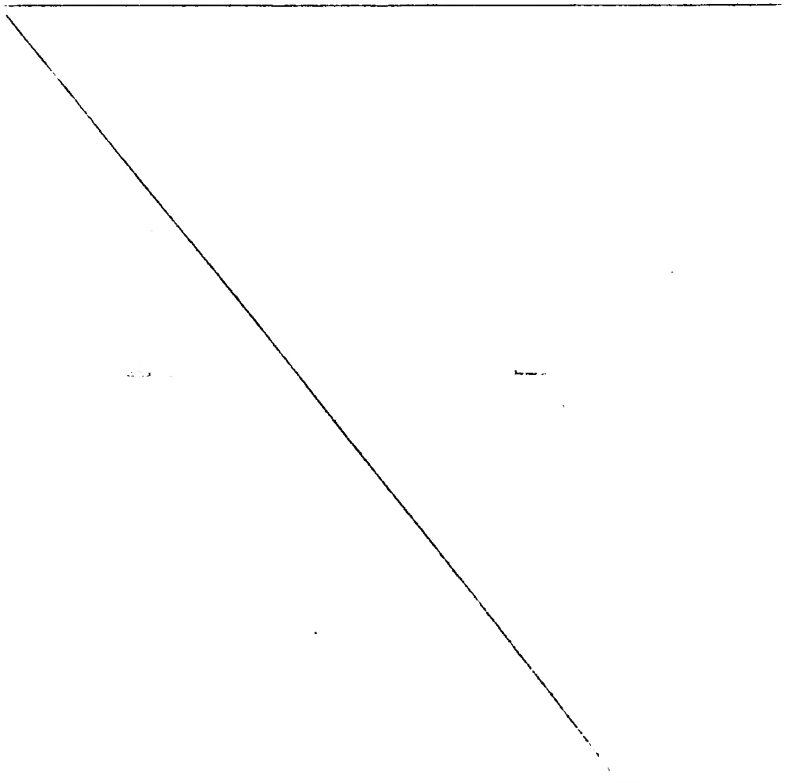
FIG. 14. Construction of authentic bGH modified with methionine at the amino terminus and various analogs of bGH.

a) Plasmid pAL401 is treated with NdeI. A synthetic DNA linker containing an ATG initiation signal and the code for the first three amino acids at the amino terminus of native bGH is ligated into the NdeI site. The resulting vector pAL601 leads to the expression of native bGH containing an extra methionine residue at the amino terminus.

b) Using the strategy described in a) but modifying the structure of the oligodeoxyribonucleotide linker a

class of vectors coding for a series of modified bovine growth hormones is constructed. The modified growth hormones start with methionine at the N-terminus and are followed by any of the twenty naturally occurring amino acids in each of positions 1 and 2, and any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp in position 3. Proceeding from position 4 to the COOH-terminus the sequence is identical to that of native bGH.

FIG. 15. Tibia test. This figure shows the comparison between effect of pRec 2/3 bGH analog and authentic bGH on the bone plate growth of hypophysectomized rats.



DETAILED DESCRIPTION OF THE INVENTION

A vector has been developed which enables the achievement of enhanced levels of gene expression and polypeptide expression. The vector is a double-stranded DNA molecule. Upon introduction into a suitable bacterial host cell containing the thermolabile repressor C_I and increasing the temperature of the host to a temperature at which the repressor is destroyed, the vector renders the host cell capable of effecting expression of a desired gene inserted into the vector and production of polypeptide encoded by the gene.

The vector includes in 5' to 3' order the following:

a DNA sequence which contains the promoter and operator P_LO_L from lambda bacteriophage;

the N utilization site for binding antiterminator N protein produced by the host cell;

a DNA sequence which contains a ribosomal binding site for rendering the mRNA of the desired gene capable of binding to ribosomes within the host cell;

an ATG initiation codon or a DNA sequence which is converted into an ATG initiation codon upon insertion of the desired gene into the vector; and

a restriction enzyme site for inserting the desired gene into the vector in phase with the ATG initiation codon.

The vector also includes a DNA sequence which contains an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell and a DNA sequence

which contains a gene associated with a selectable or identifiable phenotypic trait which is manifested when the vector is present in the host cell.

The host for use with the vector is Escherichia coli. The presently preferred strains are A1637, A1645, A2602 and A1563. A1637 is presently the most preferred strain. It was obtained from C600 by inserting transposon containing tetracycline resistance gene within the galactose operon as well as the lambda system for expression which is close to galactose operon. It has been deposited with the American Type Culture Collection in Rockville, Maryland, U.S.A. containing various plasmids as described more fully hereinafter. All such deposits were made pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms.

A1645 was obtained from A1637 by selection for Gal^+ (ability to ferment galactose) as well as loss of tetracycline resistance. It still contains the lambda expression system but part of the transposon has been removed by selection. Its phenotype is C600 r^-m^+ gal^+ thr^- leu^- lac Z^- (λcI857 Δ H1 ΔBAM N+).

A2602 and A1563 are derived from SA500. Their phenotypes are SA500 his^-ilu^- gal^+ Δ8(λCI857ΔH1ΔBAM N+ and SA500 his^- ilu^-gal^+ Δ 8 lac ZxA21 (λCI859 int2 xis1 nutL3 ΔH1), respectively.

Preferably the vector is a covalently closed circular double-stranded molecule. However, it is not essential that the vector be covalently closed.

The vector achieves its enhanced expression levels after the host cell is heated to a temperature at which the C_I

repressor is destroyed. A temperature above about 42°C is effective for this purpose and since it is desired that unnecessary heat damage to the host cells be avoided to as great an extent as possible, it is generally desirable that the temperature never exceed 42°C by more than a few degrees.

One important component of the vector is the ribosomal binding site. Suitable sites are C_{II} from lambda bacteriophage having the sequence:

TAAGGAAATACTTACAT
ATTCCTTTATGAATGTA;

a synthetic oligonucleotide having the sequence:

TAAGGAAGTACTTACAT
ATTCCTTCATGAATGTA; and

the major head protein gene of bacteriophage lambda having the sequence:

TTTTTTTACGGGATTTTTTTATG
AAAAAAATGCCCTAAAAAAATAC.

Another component of the vector is the restriction enzyme site for insertion of desired genes into the vector in phase with the ATG initiation codon. Numerous such sites may be used. The presently preferred sites are BamHl, Sacl and Nde l.

The vector also includes an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell. Suitable such origins of replication may be obtained from a number of sources. Presently preferred are

origins of replication derived from pBR322 or pR1.

A DNA sequence which contains a gene associated with a selectable or identifiable phenotypic trait which is manifested when the vector is present in the host cell is also a component of the vector. Suitable genes include those associated with temperature sensitivity or drug resistance, e.g., resistance to ampicillin, chloramphenicol or tetracycline.

Relative to vectors previously described in the scientific literature, the vectors of this invention may be used to obtain enhanced expression of a wide variety of genes encoding desirable polypeptide products. Suitable genes include those encoding growth hormones, e.g., bovine, porcine, chicken or human growth hormones; superoxide dismutase; apoprotein E; viral protein 1 of foot and mouth disease virus, protein A from S. aureus, interleukin III, enzymes, or analogs of any of the preceding. By analog is meant a polypeptide having the same activity as the naturally occurring polypeptide but having one or more different amino acids at the N-terminus of the polypeptide.

The vector may be formed by methods well known to those skilled in the art to which the invention relates. Such methods are described in greater detail in various publications identified herein, the contents of which are hereby incorporated by reference into the present disclosure in order to provide complete information concerning the state of the art.

One presently preferred vector is pMG100 having the restriction map shown in FIG. 1. This vector has had cDNA encoding bovine growth hormone inserted into its BamH1 restriction site. The resulting plasmid is designated pRec

2/3 bGH. Its restriction map is shown in FIG. 2. The plasmid pRec 2/3 bGH was introduced into Escherichia coli strain A1637 using conventional transformation methods. The resulting host vector system has been deposited under ATCC No. 39385.

A second presently preferred vector is pND5 having the restriction map shown in FIG. 3. Bovine growth hormone cDNA has been inserted into its NdeI restriction site. The resulting plasmid is designated pROll. Its restriction map is also shown in FIG. 3. The plasmid pROll was introduced into E. coli strain A1637 via transformation. The host vector system which resulted has been deposited under ATCC No. 39390.

The vector pND5 has also been used to clone human growth hormone. One plasmid designated pTV 18(1) and another designated pTV 104(2) have been created by inserting hGH cDNA into the NdeI restriction sites. pTV 18(1) is shown in FIG. 4. It has been introduced into E. coli strain A1637 via transformation. The resulting host vector system has been deposited under ATCC No. 39386. pTV 104(2) is shown in FIG. 4. It also has been introduced into E. coli strain A1637. The resulting host vector system has been deposited under ATCC No. 39384.

Using the same approach other plasmids may be prepared by inserting into the restriction enzyme site of a vector of the invention a gene encoding a desired polypeptide.

The preceding specific host vector systems involve E. coli A1637. However, as previously indicated other strains have been used including A1645, A2606 and A1563. These host vector systems may be used to produce polypeptides such as bovine and human growth hormones. To do so the host vector

system is grown under suitable conditions permitting production of the polypeptide which is then recovered.

Suitable conditions involve growth of the host vector system for an appropriate period of time at about 42°C followed by continued growth at about 37-39°C for an additional period of time, the growth being carried out on a suitable medium.

Desirably the initial period of growth is about 10 to 30 minutes at 42°C followed by growth at 37-39°C for a sufficient period of time such that the total period of growth is about 60 to 90 minutes. Preferably the growth is for about 15 minutes at 42°C followed by about 75 minutes at 38-39°C. Suitable media include lactalbumin hydrolysate with addition of glucose and brain heart infusion. In order to stably maintain the vector in the host it is critical that the host be maintained under selective pressure, e.g., addition of antibiotic.

By means of the preceding method a number of bGH and hGH analogs has been prepared. These have or may have the activity of the naturally occurring hormones.

bGH analogs have the activity of natural bGH and an identical amino acid sequence except for variations at the N-terminus of up to five (5) amino acids. Examples include the following:

1) amino acid methionine added to N-terminus of the phenylalanine form of bGH.

2) amino acid methionine added to N-terminus of the alanine form of bGH.

3) amino acid sequence Met-Asp-Gln added to N-terminus of the phenylalanine form of bGH.

4) amino acid sequence Ala-Gly added to N-terminus of the alanine form of bGH.

5) amino acid sequence Met-Gly added to N-terminus of the alanine form of bGH.

6) amino acid sequence Met-Asp-Pro-Met-Gly added to N-terminus of the alanine form of bGH.

7) amino acid sequence Met-Asp-Pro added to N-terminus of the phenylalanine form of bGH.

8) amino acid sequence Met-Thr-Arg added to N-terminus of the phenylalanine form of bGH.

9) amino acids up to methionine (4 position) removed from N-terminus of phenylalanine form of bGH.

An analog of bGH having the amino acid sequence:

$$Met\text{-}(X)_n\text{-}Y\text{-}Met...$$

wherein Met is the N-terminus, X is any of the twenty naturally occurring amino acids, Y is any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp, n is an integer from 0 to 6 and Met... is the sequence of natural bGH from position 4 to the COOH-terminus (position 191).

hGH analogs have the activity of natural hGH and an identical amino acid sequence except for variations at the N-terminus. Examples include the following:

1) amino acid methionine added to N-terminus of natural hGH.

2) amino acids up to methionine (14 position) removed from N-terminus of hGH.

An analog of hGH having the amino acid sequence:

$$Met-(X)_n-Y-Met...$$

wherein Met is the N-terminus, X is any of the twenty naturally occurring amino acids, Y is any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp, n is an integer from 0 to 13 and Met... is the sequence of natural hGH from position 14 to the COOH-terminus (position 191).

Veterinary compositions may be prepared which contain effective amounts of one or more bGH analog and a suitable carrier. Such carriers are well-known to those skilled in the art. The analogs may be administered directly or in the form of a composition to a bovine in order to increase milk or meat production.

Pharmaceutical compositions may be prepared which contain effective amounts of one or more hGH analog and a suitable carrier. Such carriers are well-known to those skilled in the art. The analogs may be administered directly or in the form of a composition to a human subject, e.g., one afflicted by dwarfism, to treat deficiencies in hGH production by the subject.

EXAMPLES

The examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be so construed as to, limit its scope in any way. The examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of genes encoding polypeptides of interest

into such vectors or the introduction of the resulting plasmids into bacterial hosts. Such methods are well-known to those skilled in the art and are described in numerous publications including the following:

Principles of Gene Manipulation, An Introduction to Genetic Engineering, 2nd Edition, edited by R.W. Old and S.B. Primrose, Univ. of Calif. Press (1981)

Met. Enzymol. vol. 68, Recombinant DNA, edited by Ray Wu.

Met. Enzymol. vol. 65, Nucleic Acids (Part 1), edited by Lawrence Grossman and Kivie Moldave

T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory, NY (1982)

H.V. Bernard et al., Gene (1979) 5, 59

A.B. Oppenheim et al., J. Mol. Biol. (1982) 158, 327

E. Remaut et al., Gene (1981) 15, 81

EXAMPLE 1
EXPRESSION VECTORS

As used herein the term expression vector refers to a group of plasmids useful for expressing desired genes in bacteria, particularly in E. coli. The desired gene may be inserted into the expression vector or alternatively, the promoters on the expression vector may be excised and placed in front of the desired gene.

I. P_L EXPRESSION VECTORS

A. pMG 100

pMG 100, as shown in FIG. 1 and described in detail under Description of the Figures is composed of λ DNA inserted into the multicopy plasmid pBR322. The salient features of the λ DNA is that it contains the λ P_L promoter, N utilization sites L and R (nut_L and nut_R) termination R1 site (t_{R1}), the C_{II} ribosomal binding site and an ATG initiation codon. Other features are shown in FIG. 1.

pMG100 was prepared from pKC30. pKC30 in turn was prepared by subcloning of λ P_L promoter in the following manner.

λ phage DNA was digested with XhoI and SmaI restriction endonucleases and the unique fragment comprised of 6393 base pairs was purified and subsequently digested with HindIII and BamH1 restriction endonucleases. The resulting fragment comprised of 2397 base pairs and containing P_L promoter was purified and ligated into a pBR322 DNA large fragment isolated from the HindIII and BamH1 digest. The subclone was identified by colony hybridization, recovered and plasmid DNA isolated

(Oppenheim, A. et al., J.Mol.Biol. (1982) 158, 327).

This plasmid and its derivatives containing eukaryotic genes may be maintained in suitable E. coli hosts. The most important feature of the host is that it provides the thermosensitive repressor CI857 and the antitermination N protein. (Gottesman, M.E. et al., J.Mol.Biol. (1978) 140, 197).

This vector has numerous advantages over previously described expression vectors including:

1. Extremely High Levels of Expression

This vector is capable of directing expression of foreign proteins in E. coli at levels as high as 15-25% of the total cellular protein.

2. Thermoinducible Regulation of Expression

The P_L promoter is inactive when the CI repressor is bound to it. The CI857 repressor is thermosensitive, that is, it binds to the promoter at 30°C but is inactived at 42°C. Thus, by increasing the temperature of fermentation to 42°C the host bacteria are induced to produce the desired protein.

The advantages of such a system include the following:

(a) a foreign protein which is toxic to E. coli can be produced when desired thus avoiding cell death early in the fermentation process.

(b) overproduction of a protein may stabilize it and prevent proteolytic degradation. (Cheng, Y.E. et al., Gene (1981) 14, 121) Thus, "instantaneous" overproduction using a tightly regulated promoter such as P_L may be preferable to continuous low level production.

3. High Copy Number

The P_L promoter in pMG100 is found on a plasmid with a high copy number in distinction to λ itself which is present in low copy numbers in E. coli. This increases expression levels.

4. Ribosome Binding Site and Initiation Codon

This expression vector contains a strong procaryotic ribosomal binding site (RBS) as well as a translation initiation codon (ATG). Thus, any eukaryotic gene may be cloned without the need for adding an initiation codon. Furthermore, the efficient RBS increases levels of expression.

5. Convenient Restriction Site

The expression vector has a BamHI site located directly following the ATG initiation codon which permits proper positioning of the desired gene in order to achieve optimal expression.

6. Nut Site

N protein which is provided by the host binds to Nut site on the expression vector and thereby prevents termination of transcription at the t_{R1} site.

B. pND5

As shown in FIG. 3, pND5 contains the P_L promoter and the other important components of the expression vectors of this invention. It includes a unique NdeI site immediately after the ribosomal binding site. The ribosomal binding site differs from the normal C_{II} site. It has the sequence:

TAAGGAAGTACTTACAT
ATTCCTTCATGAATGTA

It may be derived from a mutant or may be chemically synthesized. As described in detail under Description of the Figures pND5 was derived from pOG7. (Oppenheim, A., et al., J.Mol.Biol. (1982) 158, 327) This vector does not contain a translation initiation codon. It appears to provide superior expression of modified bGH and hGH, particularly enhanced yield relative to pMG100 containing a bGH analog.

C. pND55

pND55 is a derivative of pND5 which contains the convenient restriction site SacI in front of C_{II}-RBS and ATG initiation codon. Cleavage of the plasmid at this site and subsequent treatment with DNA polymerase Klenow fragment allows one to obtain an ATG initiation codon to which any desired gene can be ligated. (FIG. 3 and Description of FIG. 3.)

II. TRP EXPRESSION VECTORS

A. pAL Trp 46

pAL Trp 46 contains the Trp promoter and the first seven amino acids of the Trp E gene fused to the β-galactosidase gene. (FIG. 5). The desired gene can be inserted into a BamHI site which follows the 7 amino acids of Trp E.

B. pAL Trp 47; Trp 46 Deleted of Attenuator

This is a construction based on Trp 46 in which the attenuator region of the Trp promoter has been deleted.

C. Trp-Lac Fusions

The construction of this promoter, found on plasmid p4754 is illustrated in FIGS. 6 and 7. A variation of this construction is outlined in FIG. 8.

III. Histidine Promoter Expression Vectors

The construction of this expression vector is illustrated in FIGS. 9 and 10.

IV. Other Promoters Used

A. Lac

This promoter was used in the construction of pYL 301 as shown in FIG. 11.

B. Omp F

This is a promoter system which expresses a protein attached to a signal sequence. The signal sequence is removed when the protein is translocated across the membrane. (FIG. 12)

EXAMPLE 2

Bovine Growth Hormone

The starting point for bGH cDNA modifications is plasmid D_4 which has been described previously. (Keshet, E. et al, Nucleic Acids Research (1981) 9, 19). The D_4 plasmid is also described in pending U.S. patent application, Serial No. 245,943, filed March 20, 1981, claiming priority of Israel patent application, Serial No. 59,690 filed March 24, 1980. It has previously been deposited with the American Type Culture Collection in an E. coli host under ATCC No. 31826.

I. pRec 2/3 bGH

The construction of pRec 2/3 is shown in FIG. 2 and described in the Description of the Figures. bGH cDNA from D_4 has been manipulated prior to insertion into PMG100 to provide the correct reading frame.

pRec 2/3 has been introduced into various E. coli strains including A1637 by transformation using known methods. A1637 containing pRec 2/3 has been deposited under ATCC No. 39385. This strain produces upon growth and induction an analog of bGH having the amino acid sequence Met-Asp-Gln added to the N-terminus of the phenylalanine form of natural bGH. The amount of bGH analog produced by pRec 2/3 was about 23% of the total protein produced by the bacteria as calculated from scanning of Coomasie stained SDS polyacrylamide gels.

II. pRO11

The construction of pRO11 is shown in FIG. 3 and described in the Description of the Figures. The pND5 vector DNA is restricted with Ndel. Insertion of the Ndel fragment from

pRec 2/3 (FIG. 2) which contains bGH cDNA results in the plasmid pRO11.

pRO11 has been introduced into E. coli A1637 by transformation. The resulting host vector system has been deposited under ATCC No. 39390. This strain when grown and induced produces the same analog as pRec 2/3. Preliminary results indicate that pRO11 produces up to 20% more bGH analog than pRec 2/3. The methods used to grow the strain, recover the bGH analog produced and purify it are the same as those described for pRec 2/3 in Example 4.

III. pAL401

The construction of pAL401 is shown in FIG. 13 and described in the Description of the Figures. bGH cDNA from D_4 by way of pAL-500 (FIG. 2) was inserted into pND11 as shown in FIG. 13.

pAL401 may be introduced into E. coli A1637 by transformation. The resulting strain produces an analog of bGH in which Met^4 of natural bGH is at the N-terminus and the amino acids preceding Met^4 have been deleted.

IV. pAL601

The construction of pAL601 is shown in FIG. 14 and described in the Description of the Figures. It is a derivative of pAL401 (FIG. 13).

pAL601 may be introduced into E. coli A1637 by transformation. The resulting strain produces an analog of bGH in which Met has been added to the N-terminus of the phenylalanine form of bGH.

EXAMPLE 3

Human Growth Hormone

The starting point for hGH cDNA was cloning of the cDNA from mRNA purified from hypophyses tumor from acromegalic patients into the HindIII site of pBR322.

I. pTV 18(1)

The construction of PTV 18(1) is shown in FIG. 4 and described in the Description of the Figures. hGH cDNA was manipulated prior to insertion into pND5 to provide the correct reading frame.

pTV 18(1) was introduced into E. coli A1637 by transformation. The resulting bacteria have been deposited under ATCC No. 39386. This strain upon growth and induction produces an analog of hGH having the sequence of natural hGH beginning with Met^{14} and lacking amino acids 1-13. The amount of hGH analog produced by pTV 18(1) was about 8% of the total protein produced by the bacteria.

II. pTV 104(2)

The construction of pTV 104(2) is shown in FIG. 4 and described in the Description of the Figures. hGH cDNA was manipulated prior to insertion into pND5 to provide the correct reading frame.

pTV 104(2) was introduced into E. coli A1637 by transformation. The resulting bacteria have been deposited under ATCC No. 39384. This strain upon growth and induction produces an analog of hGH having the sequence of natural hGH preceded by Met at the N-terminus. The amount of hGH analog produced by pTV 104(2) was above 25% of the total protein produced by the bacteria.

EXAMPLE 4

Growth of pRec 2/3

Stock Cultures: Stock cultures of pRec 2/3 in A1637 are grown on BHI medium (see inoculum), then diluted twofold with 87% glycerol containing phosphate citrate buffer, and stored at -70°C.

Inoculum: Inoculum is propagated in BHI medium (37 g/l) brain heart infusion (DIFCO). Sterile medium in shake flask is inoculated from stock culture and incubated 15 hours on shaker at 30°C, 200 r.p.m. Subsequent stages in inoculum propagation are carried out in stirred aerated fermentors. Sterile medium is inoculated with 0.2 ml flask culture per 1, and incubated 15 hours at 30°C, pH 7 ± 0.5 with agitation and aeration to maintain dissolved oxygen level above 20% air saturation.

Production: Production medium contains:

Lactalbumin hydrolysate (enzymatic)	20 g/l
Yeast extract	10 g/l
K_2HPO_4	2.5 g/l
NaCl	10 g/l
Ampicillin	0.1 g/l
Biotin	0.1 mg/l
Thiamine	1 mg/l
Trace elements solution	3 ml/l

Ampicillin, biotin and thiamine in solution are filter sterilized separately and added to the sterile production medium before inoculation. Sterile glucose solution is added initially to supply 10 g/l, and during the induction and expression procedure to maintain glucose above 10 g/l

Trace elements solution contains:

$MgSO_4 \cdot 7H_2O$	170 g/l
$FeCl_3$	16 g/l
$ZnCl_2 \cdot 4H_2O$	2 g/l
$CoCl_2 \cdot 6H_2O$	2 g/l
$Na_2MoO_4 \cdot 2H_2O$	2 g/l
$CaCl_2 \cdot 2H_2O$	1 g/l
$CuCl_2$	1 g/l
H_3BO_3	0.5 g/l
Conc. HCl	100 ml/l

The medium is inoculated with 5-10% inoculum culture and incubated at 30°C. Agitation-aeration rates are set to maintain dissolved oxygen level above 20% air saturation. The pH is maintained at 7 ± 0.2 with NH_3. Once cell concentration reaches about 3 g/l (OD_{660} = 10) induction is started.

Temperature is raised to 42°C. Maintained there for 15 minutes, then lowered to 38°C. Following incubation at 38°C for 1- 1 1/2 hours, the culture is chilled, and cells are recovered by centrifugation for hormone purification.

Recovery of bGH

One kilogram of bacterial cells is suspended in 10 volumes of the solution containing 50 mM Tris-Cl (pH 7.4), 50 mM EDTA and 25% sucrose in a Warring blender, with a control of blender's speed to minimize foaming. The homogeneous suspension is continuqusly passed through a Dynomill cell disruptor (Willy A. Bachofen, Basel) and the homogeneous suspension of disrupted cells is clarified first by cen-

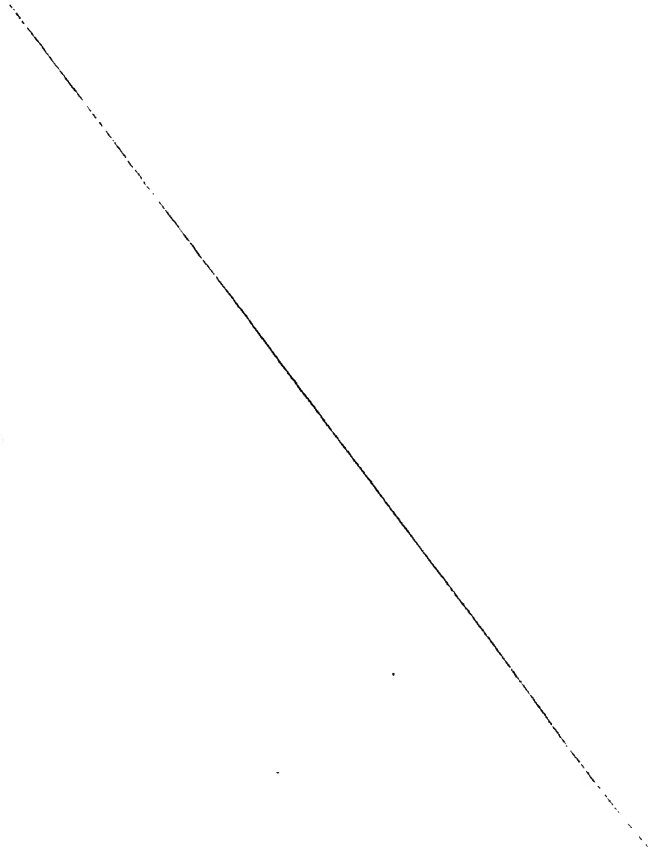
trifugation in a Sharpless centrifuge followed by a continuous centrifugation at 20,000 rpm in a Sorvall centrifuge. The precipitate from both centrifugation steps is collected, washed with 50 mM Tris-Cl (pH 7.4) and resuspended in 500 ml of the same buffer. Lysozyme is added to a final concentration of 2 mg/ml and the suspension is incubated for 1 hour at 37°C. Triton X-100 is then added to a final concentration of 1%, the suspension is cooled to 4°C and centrifuged at 20,000 rpm for 20 minutes in a Sorvall SS34 rotor. The precipitate is collected, washed twice with 50 mM Tris-Cl, resuspended in 500 ml of 50 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$ and deoxyribonuclease is added to a final concentration of 20 µg/ml. After incubation for 30 minutes at room temperature the precipitate is collected as above, washed twice with 500 ml of 20 mM Tris-Cl (pH 7.4), 100 mM NaCl and 10 mM EDTA, followed by two washings with 500 ml of distilled water. The precipitate is collected by centrifugation and can be stored at -20°C for an indefinite time. At this stage the bGH is 80% pure as judged by sodium dodecyl sulfate-gel electrophoresis. The yield is approximately 15 g of bGH.

Purification of bGH

One hundred gr of precipitate is suspended in 40 ml distilled water and solubilized by titration with 0.5 M NaOH, pH 11.8. The solution is then sonicated for 2 minutes and clarified by centrifugation at 20,000 rpm in a Sorvall SS34 rotor for 20 minutes. The solution is then applied onto a Sepharose CL-6B column (5 x 100 cm) equilibrated with 6.5 mM borate buffer, pH 11.8. Column is developed at the rate of 100 ml/hr and fractions of 12 ml are collected. The first peak off the column is discarded. The following two peaks are separated and pooled. The first represents aggregated bGH with low activity; the second bGH with high

activity.

A DEAE-Sephacel (25 g/100 gr. equiv. ppt) column is equilibrated with 6.5 mM borate buffer, pH 9.0. The second bGH peak is brought to pH 9.0 with HCl loaded on the DEAE Sephacel column at a rate of 250 ml/hr. The column is washed with 7.5 ml of 6.5 mM borate buffer, pH 9.0, eluted with 6.5 mM borate buffer, pH 9.0 containing 75 mM NaCl. The fractions with OD_{280} above 0.3 are pooled, dialysed against H_2O in Millipore Pellicon dialysis apparatus and then lyophilized.



EXAMPLE 5

Activity of bGH Analog Produced by pRec 2/3

1. Radioimmunoassay comparison of bGH analog with natural bGH

A solution containing 100 ng/ml bGH analog was prepared in phosphate buffered saline (1% BSA). This solution was diluted serially to concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 ng/l. Duplicate 0.1 ml aliquots of these solutions were submitted to RIA using a double antibody procedure. The dilution curve was comparable to that obtained with natural bGH.

2. Radioreceptor binding Assay

A radioreceptor binding assay was performed with rabbit liver membranes as described by T. Tushima and H.G. Freisen (Y. Chin., Endocr. Metab. (1973) 37, 334 using ^{125}I-hGH as the tracer and authentic bGH solutions for the construction of calibration curves. Samples were incubated in triplicate for two hours at room temperature in 0.3 ml of assay buffer (50 mM Tris, 15 mM $CaCl_2$ and 5 mg/ml bovine serum albumin, pH 7.6). The tubes contained ^{125}I-hGH (20,000 cpm of preparation of 30-60 µci/µg), 150-250 µg liver membrane protein and either natural bGH (1-100 ng) or extracts of bacterial bGH. The result demonstrated that the bGH activity of the bGH analog is comparable to that of natural bGH.

3. Tibia Test

The bioactivity of the pRec 2/3 bGH analog recovered from engineering bacterial cells according to Example 4 was evaluated by a tibia test. (Parlow, A.F., et al., Endocrinology (1965) 77, 1126.)

Rats were hypophysectomized at 28-30 days of age, then kept for 10-14 days without treatment. Bovine growth hormone derived from bovine pituitaries or from recombinant E. coli was dissolved in 0.15M NaCl + 0.01 M borate, pH 10.0. Rats (4-7 per group) received daily subcutaneous injections of bGH solutions (5-125 µg/day in 0.2 cc) for 5 days while kept on a normal diet (Purina Rat-Chow and water ad-libitum). The animals were sacrificed on the 6th day, their foreleg knee-bones taken out, cut longitudinally, fixed with acetone and stained with 2% $AgNO_3$. The width of the epiphyseal plates were measured by observation through a dissecting binocular (Nikon). Mean values (of 40 readings per rat) were used for the construction of log dose-response curves. Results are shown in FIG. 15.

EXAMPLE 6

bGH Analogs

Table I sets forth a series of plasmids which have been constructed and the analogs which were produced from them.

TABLE I

PLASMID	AMINO TERMINUS OF bGH ANALOGS
Rec 2/3	Met Asp Gln Phe[2]
pB 1	Met Asp Pro Met Gly Ala Phe[2]
pM 4	Met Asp Pro Phe[2]
pM 1	Met Ala[1] Phe[2]
pM 2	Met Ala[1] Phe[2]
pAL 401	Met[4]
pYL 301	Met Gly Ala[1] Phe[2]
pAL 302	11 A.A + Ala[1] Phe[2]
pHis 129	Met Thr Arg Phe[2]
pAL 312	Met Gly Ala[1] Phe[2]
pAL 322	Met Gly Ala[1] Phe[2]
pAL 601R	Met Gly Ala[1] Phe[2]
p 18	Met Gly Ala[1] Phe[2]
PBTG-800	Met Glu Phe[2]
pORF 2-12	Ala Gly Ala[1] Phe[2]

EXAMPLE 7

Effect of pRec 2/3 bGH analog on Lactogenesis in Dairy Cows

The lactogenic effect of bGH has been well documented in the scientific literature in the reports of Bines, J. et al, Brit J. Nutri. (1980) 43, 179 and Peel, C. et al, J. Nutr. (1981) 111, 1662. Bauman, D. et al, J. Dairy Sci. Vol. Supp. 1, Abst 86 (1982) reported that milk production was increased by rDNA bGH. An experiment was conducted to determine the effects of pRec 2/3 bGH on lactogenesis in comparison with natural bGH. Eighteen Holstein cows ranging from 141 to 154 days postpartum were randomly assigned to treatment and blocked according to milk production according to the following design.

Pretreatment	Treatment	Daily GH Injection
Control	5 days	Saline
Natural bGH	5 days	25 mg/day for 10 days
pRec 2/3 bGH	5 days	25 mg/day for 10 days

The bGHs were put in solution with 0.1 M $NaHCO_3$ aqueous buffer (pH = 8.2) at the concentration of 1 mg/ml immediately prior to each day's injections. The cows were injected with placebo or bGH solution daily for 10 days in a subcutaneous site in the neck region. No injections were given during the 5-day pretreatment period.

The cows were milked twice daily at approximately 6:00 a.m. and 5:00 p.m. Milk weights were recorded by the Boumatic system and recorded in the dairy data system.

The average milk production values for the pretreatment and bGH treatment periods are shown in Table II. The production level of the control cows was unchanged while

the milk volume increased to a similar degree in both the bGH groups. The natural bGH caused an 11.9% increase in milk for a 10-day period and bGH analog treatment resulted in a 10.2% increase. The data were not analyzed for statistical significance due to the small number of animals, however, the magnitudes of the increases are similar to those reported in the literature.

It was concluded that pRec 2/3 bGH stimulates lactogenesis in dairy cows similar to natural bGH.

TABLE II

Bovine Growth Hormone Effect on Lactogenesis
Natural bGH vs pRec 2/3 bGH

Treatment Group	No.	Av. Daily Milk Production lb/day		
		Pretreatment 5 days	During GH 10 days	% Increase Over Pretreatment
Control	6	57.23	57.26	-
Natural bGH 25 mg/day	5	58.54	65.50	11.9
pRec 2/3 bGH 25 mg/day	6	57.48	63.34	10.2

Each cow was injected daily subcutaneously with either placebo or bGH solution once daily for 10 days.

WHAT IS CLAIMED IS:

1. An improved vector which upon introduction into a suitable bacterial host cell containing the thermolabile repressor C_I renders the host cell capable, upon increasing the temperature of the host cell to a temperature at which the repressor is destroyed, of effecting expression of a desired gene inserted into the vector and production of polypeptide encoded by the gene comprising:

a double-stranded DNA molecule which includes in 5' to 3' order the following:

a DNA sequence which contains the promoter and operator P_LO_L from lambda bacteriophage;

the N utilization site for binding antiterminator N protein produced by the host cell;

a DNA sequence which contains a ribosomal binding site for rendering the mRNA of the desired gene capable of binding to ribosomes within the host cell;

an ATG initiation codon or a DNA sequence which is converted into an ATG initiation codon upon insertion of the desired gene into the vector; and

a restriction enzyme site for inserting the desired gene into the vector in phase with the ATG initiation codon;

and which additionally includes a DNA sequence which contains an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell and a DNA sequence which contains a gene associated with a selectable or identifiable phenotypic trait which is manifested when

the vector is present in the host cell.

2. A vector of claim 1, wherein the suitable host cell is Escherichia coli.

3. A vector of claim 2, where the Escherichia coli is strain A1637, A1645, A2602 or A1563.

4. A vector of claim 1, wherein the double-stranded DNA molecule is circular.

5. A vector of claim 1, wherein the temperature is above about 42°C.

6. A vector of claim 1, wherein the ribosomal binding site is C_{II} from lambda bacteriophage and has the sequence:

TAAGGAAATACTTACAT
ATTCCTTTATGAATGTA

7. A vector of claim 1, wherein the ribosomal binding site is a synthetic oligonucleotide having the sequence:

TAAGGAAGTACTTACAT
ATTCCTTCATGAATGTA

8. A vector of claim 1, wherein the ribosomal binding site is from the major head protein gene of bacteriophage lambda and has the sequence:

TTTTTTTACGGGATTTTTTTATG
AAAAAAATGCCCTAAAAAAATAC

9. A vector of claim 1, wherein the restriction enzyme site is BamHl, Sacl or Ndel.

10. A vector of claim 1, wherein the origin of replication is derived from pBR322.

11. A vector of claim 1, wherein the origin of replication is derived from pR1.

12. A vector of claim 1, wherein the phenotypic trait is drug resistance or temperature sensitivity.

13. A vector of claim 12, wherein the drug resistance is resistance to ampicillin, chloramphenicol or tetracycline.

14. A vector of claim 1, wherein the desired gene encodes a growth hormone, superoxide dismutase, apolipoprotein E, viral protein 1 of foot and mouth disease virus, protein A from S. aureus, interleukin III, an enzyme or analogs thereof.

15. A vector of claim 1, wherein the desired gene encodes bovine growth hormone or analogs thereof.

16. A vector of claim 1, wherein the desired gene encodes human growth hormone or analogs thereof.

17. A vector of claim 1, wherein the desired gene encodes porcine growth hormone or analogs thereof.

18. A vector of claim 1, wherein the desired gene encodes chicken growth hormone or analogs thereof.

19. The vector pMG100 having the restriction map shown in FIG. 1 and deposited under ATCC No. 39385 with bGH cDNA cloned into BamH1 restriction site as shown in FIG. 2.

20. The vector pND5 having the restriction map shown in

FIG. 3 and deposited under ATCC Nos. 30394 and 39386 with hGH cDNA cloned into Ndel restriction sites as shown in FIG. 4.

21. A plasmid for production of a polypeptide which comprises the vector of claim 1 and a gene encoding the polypeptide or an analog thereof inserted into the restriction enzyme site.

22. A plasmid for production of bovine growth hormone which comprises the vector of claim 1 and a gene encoding bovine growth hormone or an analog thereof inserted into the restriction enzyme site.

23. The plasmid of claim 22 designated pRec 2/3 bGH having the restriction map shown in FIG. 2 and deposited under ATCC No. 39385.

24. The plasmid of claim 22 designated pRO11 having the restriction map shown in Fig. 3 and deposited under ATCC No. 39390.

25. A plasmid for production of human growth hormone which comprises the vector of claim 1 and a gene encoding human growth hormone or an analog thereof inserted into the restriction enzyme site.

26. A plasmid of claim 25 designated pTV 18(1) having the restriction map shown in FIG. 4 and deposited under ATCC No. 39386.

27. A plasmid of claim 25 designated pTV 104(2) having the restriction map shown in FIG. 4 and deposited under ATCC No. 39384.

28. A host vector system for production of polypeptide comprising the plasmid of claim 21 in a suitable host.

29. A host vector system of claim 28, wherein the host is A1637, A1645, A2602 or A1563.

30. A host vector system for production of bovine growth hormone comprising the plasmid of claim 22 in a suitable host.

31. The host vector system of claim 30, wherein the host is A1637, A1645, A2602 or A1563.

32. A host vector system for production of bovine growth hormone comprising the plasmid of claim 23 in a suitable host.

33. A host vector system for production of bovine growth hormone comprising the plasmid of claim 24 in a suitable host.

34. A host vector system for production of human growth hormone which comprises the vector of claim 25 in a suitable host.

35. A host vector system of claim 34, wherein the host is A1637, A1645,A2602 or A1563.

36. A host vector system for production of human growth hormone comprising the plasmid of claim 26 in a suitable host.

37. A host vector system for production of human growth hormone comprising the plasmid of claim 27 in a suitable host.

38. A method for producing a polypeptide which comprises growing the host vector system of claim 28 under suitable conditions permitting production of the polypeptide and recovering the resulting polypeptide.

39. A method of claim 38, wherein the suitable conditions comprise growth of the host vector system for an appropriate period of time at about 42°C followed by continued growth at about 37-39°C for an additional period of time, said growth being carried out on a suitable medium.

40. A method of claim 39, wherein the appropriate period of time at 42°C is about 10 to 30 minutes and the additional period of time at 37-39°C is sufficient to make the total period of growing time about 60 minutes to 90 minutes.

41. A method of claim 40, wherein the appropriate period of time at 42°C is about 15 minutes and the additional period is about 75 minutes at 38-39°C.

42. A method of claim 39, wherein the suitable medium is lactalbumin hydrolysate with addition of glucose or brain heart infusion.

43. A method for producing bovine growth hormone which comprises growing the host vector system of claim 32 under suitable conditions permitting production of bGH and recovering the resulting bGH.

44. A method for producing bovine growth hormone which comprises growing the host vector system of claim 33 under suitable conditions permitting production of bGH and recovering the resulting bGH.

45. A method for producing human growth hormone which

comprises growing the host vector system of claim 36 under suitable conditions permitting production of hGH and recovering the resulting hGH.

46. A method for producing human growth hormone which comprises growing the host vector system of claim 37 under suitable conditions permitting production of hGH and recovering the resulting hGH.

47. An analog of bGH having the activity of naturally occurring bGH and a similar amino acid sequence varying from the sequence of natural bGH by up to 5 amino acids at the N-terminus.

48. An analog of claim 47 having the amino acid methionine at the N-terminus of the phenylalanine form of bGH.

49. An analog of claim 47 having the amino acid methionine at the N-terminus of the alanine form of bGH.

50. An analog of claim 47 having the amino acid sequence Met-Asp-Gln at the N-terminus of the phenylalanine form of bGH.

51. An analog of claim 47 having the amino acid sequence Ala-Gly at the N-terminus of the alanine form of bGH.

52. An analog of claim 47 having the amino acid sequence Met-Gly at the N-terminus of the alanine form of bGH.

53. An analog of claim 47 having the amino acid sequence Met-Asp-Pro-Met-Gly at the N-terminus of the alanine form of bGH.

54. An analog of claim 47 having the amino acid sequence

Met-Asp-Pro at the N-terminus of the phenylalanine form of bGH.

55. An analog of bGH having the amino acid sequence Met-$(X)_n$-Y-Met... wherein Met is the N-terminus, X is any of the twenty naturally occurring amino acids, Y is any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp, n is an integer from 0 to 6 and Met... is the sequence of natural bGH from position 4 to the COOH-terminus (position 191).

56. An analog of claim 47 having the amino acid sequence of the phenylalanine form of bGH after removal of the amino acids up to methionine (4 position) at the N-terminus.

57. An analog of claim 47 having the amino acid sequence Met-Thr-Arg at the N-terminus of the phenylalanine form of bGH.

58. An analog of hGH having the activity of naturally occurring hGH and a similar amino acid sequence varying from the sequence of natural hGH.

59. An analog of claim 58 having the amino acid sequence of hGH after removal of the amino acids up to methionine (14 position) at the N-terminus.

60. An analog of claim 58 having the amino acid methionine at the N-terminus of natural hGH.

61. A veterinary composition comprising an effective amount of an analog of bGH in accordance with claim 47 and a suitable carrier.

62. A veterinary composition comprising an effective

amount of an analog of bGH in accordance with claim 50 and a suitable carrier.

63. A pharmaceutical composition comprising an effective amount of an analog of hGH in accordance with claim 58 and a suitable carrier.

64. A method of stimulating milk or meat production in a bovine which comprises administering to the bovine an effective amount of the bGH analog of claim 47.

65. A method of stimulating milk or meat production in a bovine which comprises administering to the bovine an effective amount of the bGH analog of claim 50.

66. A method of treating human growth hormone deficiency which comprises administering to a subject afflicted thereby an effective amount of the hGH analog of claim 58.

67. An analog of hGH having the amino acid sequence Met-$(X)_n$-Y-Met... wherein Met is the N-terminus, X is any of the twenty naturally occurring amino acids, Y is any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp, n is an integer from 0 to 13 and Met... is the sequence of natural hGH from position 14 to the COOH-terminus (position 191).

68. A method for recovering a purified animal growth hormone or an analog thereof from a bacterial cell in which the animal growth hormone has been produced by means of expression of a plasmid containing a DNA sequence encoding the hormone or analog which comprises:

a. disrupting the cell wall of the bacterial cell to produce a lysate;

b. adjusting the pH of the lysate to a neutral pH so as to precipitate the hormone or analog;

c. solubilizing the precipitate by adjusting the pH to an alkaline pH.

d. separating the solubilized hormone or analog precipitate from other soluble components by gel filtration chromatography; and

e. subjecting the hormone or analog thus separated to ion exchange chromatography to concentrate the hormone or analog and thereby recovering purified hormone or analog.

69. A method of claim 68, wherein the hormone or analog is bovine growth hormone or an analog thereof.

70. A method of claim 68, wherein the hormone or analog is porcine growth hormone or an analog thereof.

71. A method of claim 68, wherein the hormone or analog is chicken growth hormone or an analog thereof.

72. A method of claim 68, wherein the hormone or analog is

human growth hormone or an analog thereof.

73. A method of claim 68, wherein the cells are mechanically disrupted.

74. A method of claim 68, wherein lysozyme is added to the lysate prior to adjusting the pH.

75. A method of claim 68, wherein deoxyribonuclease is added to the lysate prior to adjusting the pH.

76. A method of claim 68, wherein the neutral pH is about 7.4.

77. A method of claim 68, wherein the alkaline pH is about 11.8.

78. A method of claim 68, wherein the hormone or analog is further concentrated by dialysis followed by lyophilization.

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FIG.1.

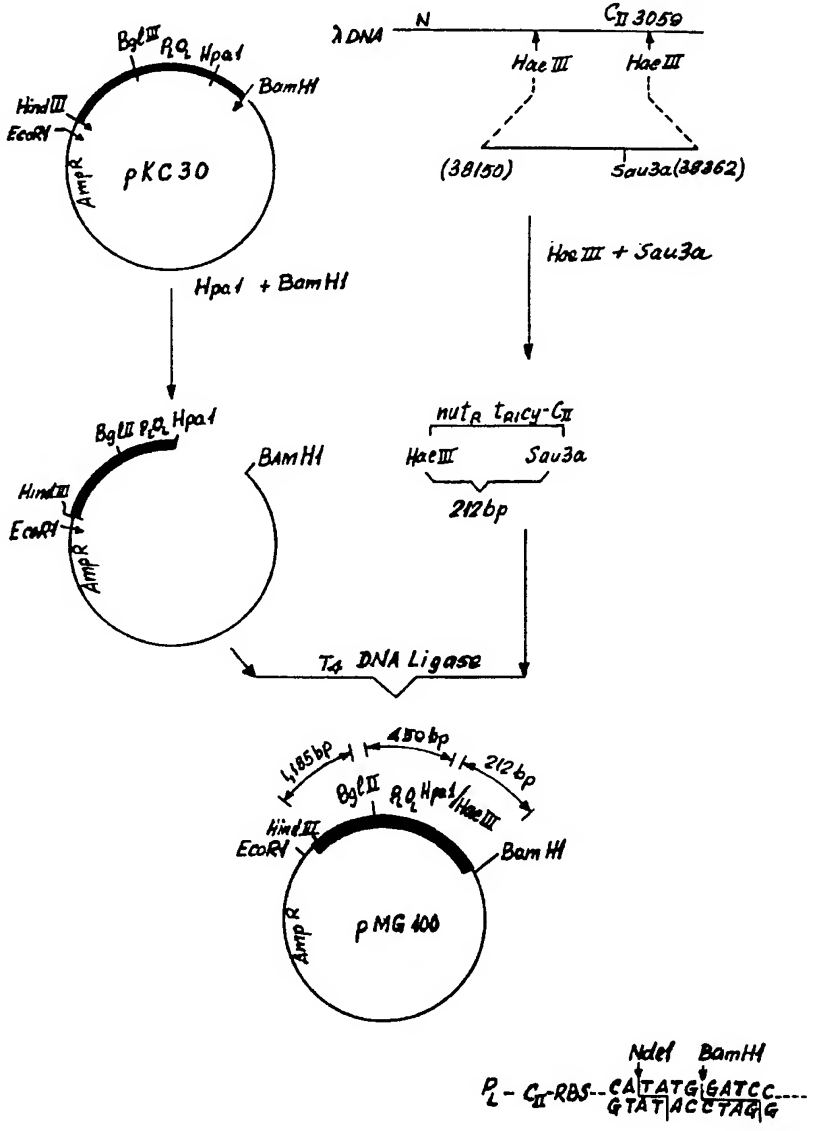

FIG.2.

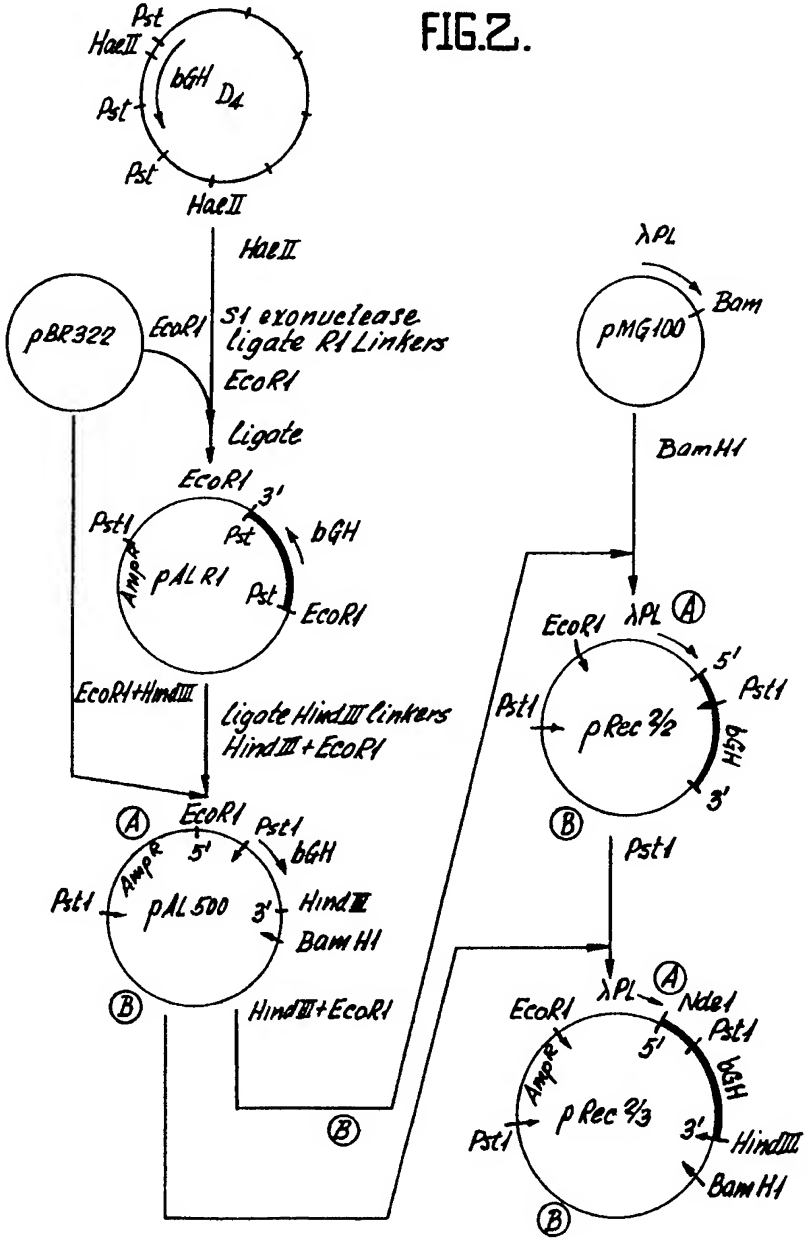
Pst
HaeII
bGH
D4
Pst
Pst
HaeII
HaeII
pBR322
EcoR1
S1 exonuclease
ligate R1 Linkers
EcoR1
Ligate
EcoR1
3'
Pst1
Pst
bGH
Amp^R
pAL R1
Pst
EcoR1
EcoR1+HindIII
Ligate HindIII Linkers
HindIII + EcoR1
A
EcoR1
Pst1
5'
bGH
Amp^R
Pst1
pAL500
3'
HindIII
BamH1
B
HindIII+EcoR1
B
λPL
pMG100
Bam
BamH1
EcoR1
λPL
A
5'
Pst1
Pst1
pRec 3/2
H59
3'
B
Pst1
λPL
A
Nde1
EcoR1
Pst1
5'
Amp^R
bGH
pRec 2/3
3'
HindIII
Pst1
BamH1
B

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FIG.3.

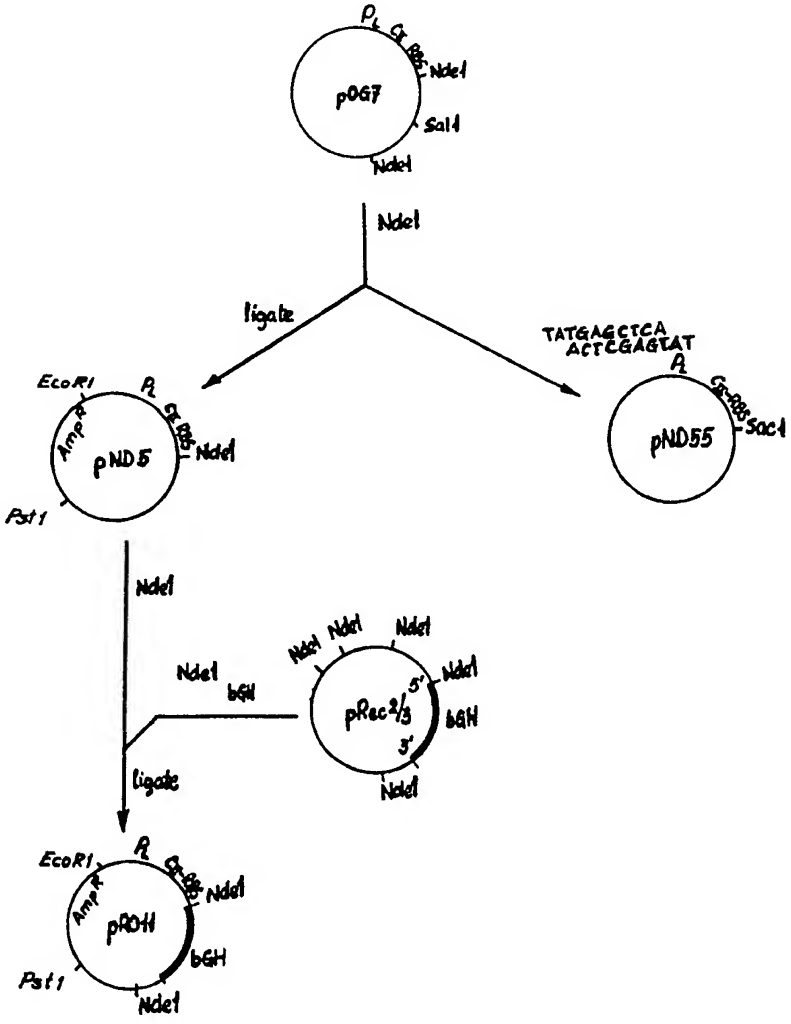

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FIG4.

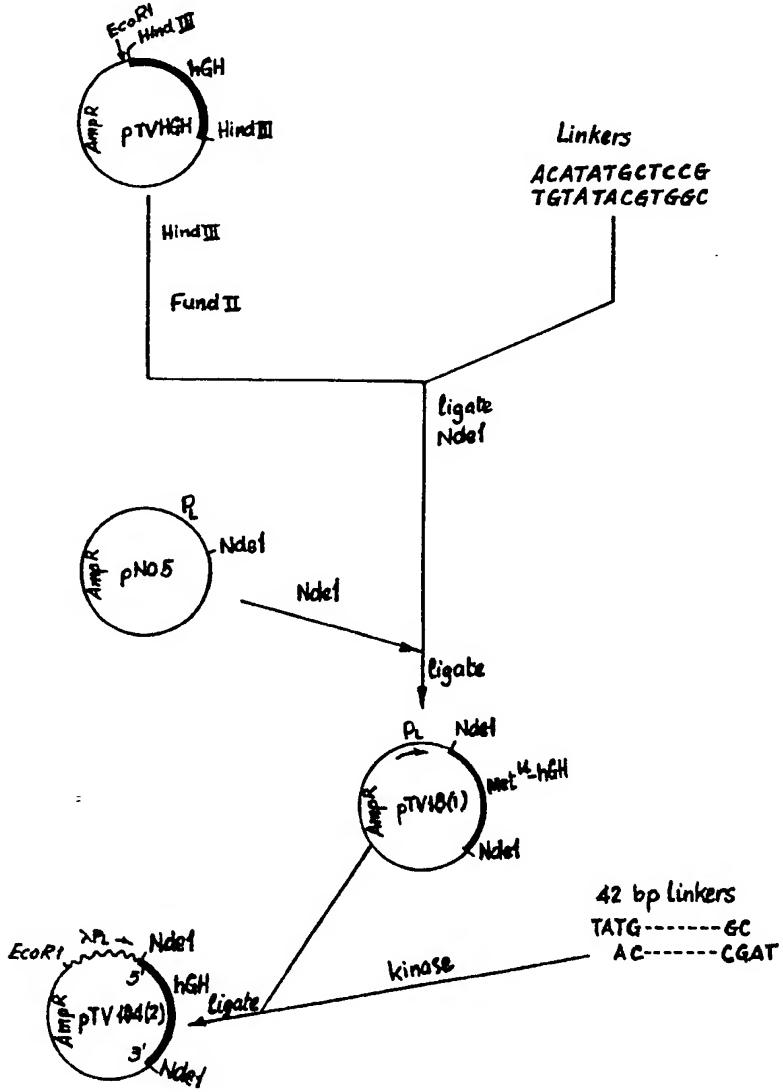

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FIG.5.

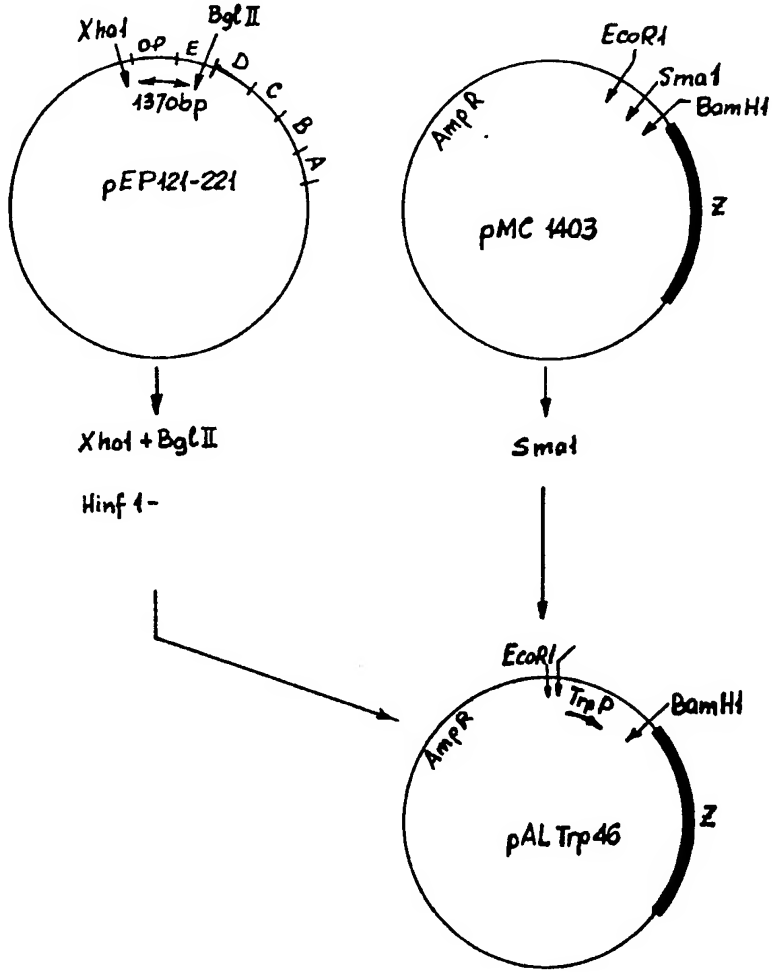


FIG.6.

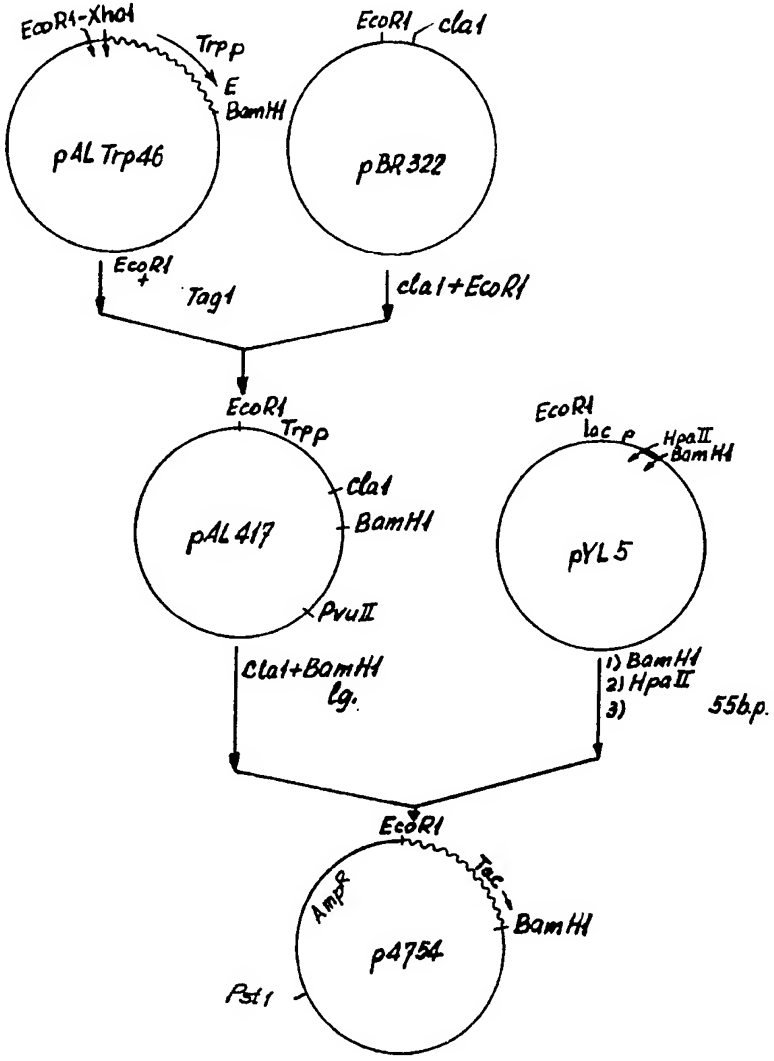
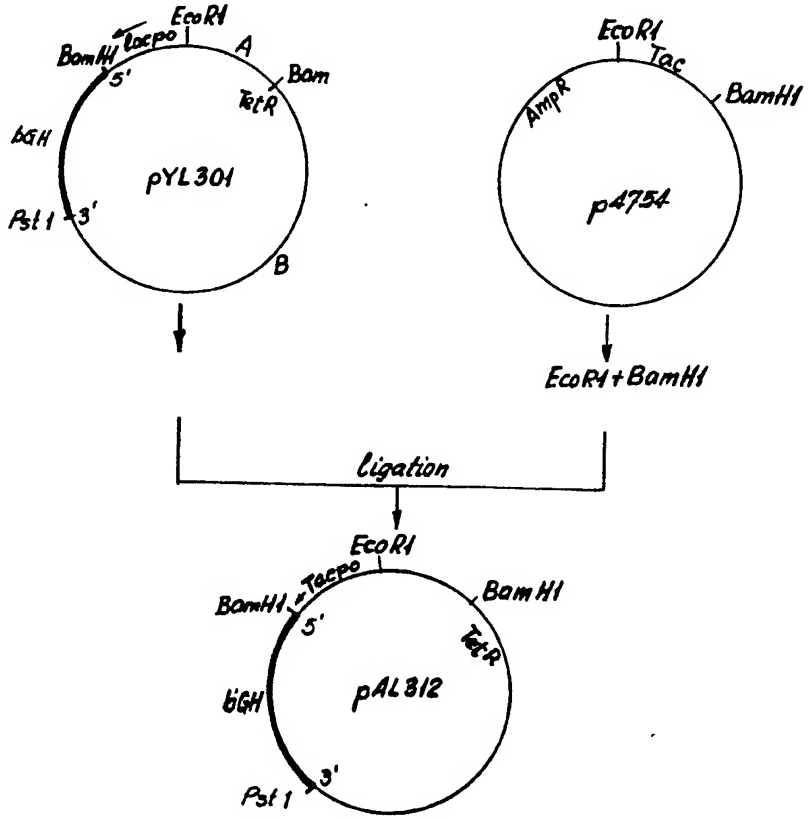

EcoR1-Xho1
Trp P
E
BamH1
pAL Trp46
EcoR1
cla1
pBR322
EcoR1
+
Tag1
cla1+EcoR1
EcoR1
Trp P
cla1
BamH1
PvuII
pAL 417
EcoR1
lac P
HpaII
BamH1
pYL5
Cla1+BamH1
lg.
1) BamH1
2) HpaII
3)
55b.p.
EcoR1
Amp R
Tac
BamH1
Pst1
p4754

FIG.7.



CACACAGGAAACAG GAT CCT ATG GGC GCC TTC

S.D.

fMet Gly Ala Phe

FIG.8.

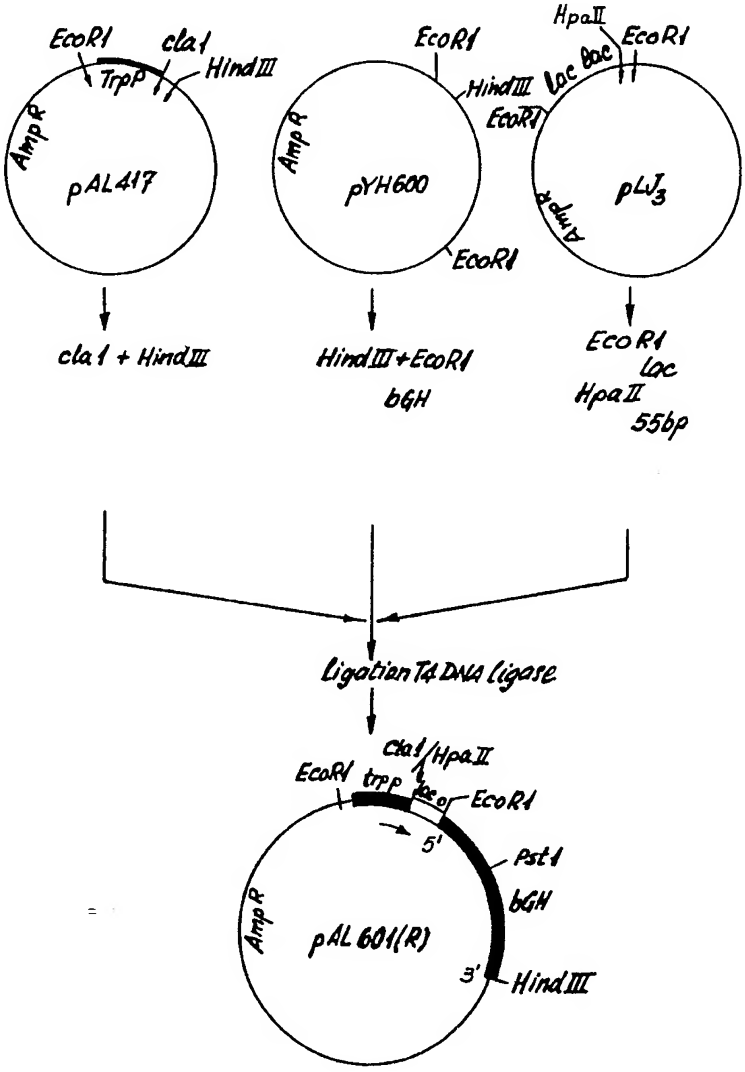

EcoR1
cla1
HindIII
TrpP
AmpR
pAL417
cla1 + HindIII
EcoR1
HindIII
AmpR
pYH600
EcoR1
HindIII+EcoR1
bGH
HpaII
EcoR1
Lac
HindIII
EcoR1
Lac
AmpR
pLJ3
EcoR1
Lac
HpaII
55bp
Ligation T4 DNA Ligase
Cla1/HpaII
EcoR1
trpp
Lac o
EcoR1
5'
Pst1
bGH
AmpR
pAL601(R)
3'
HindIII

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FIG.9.

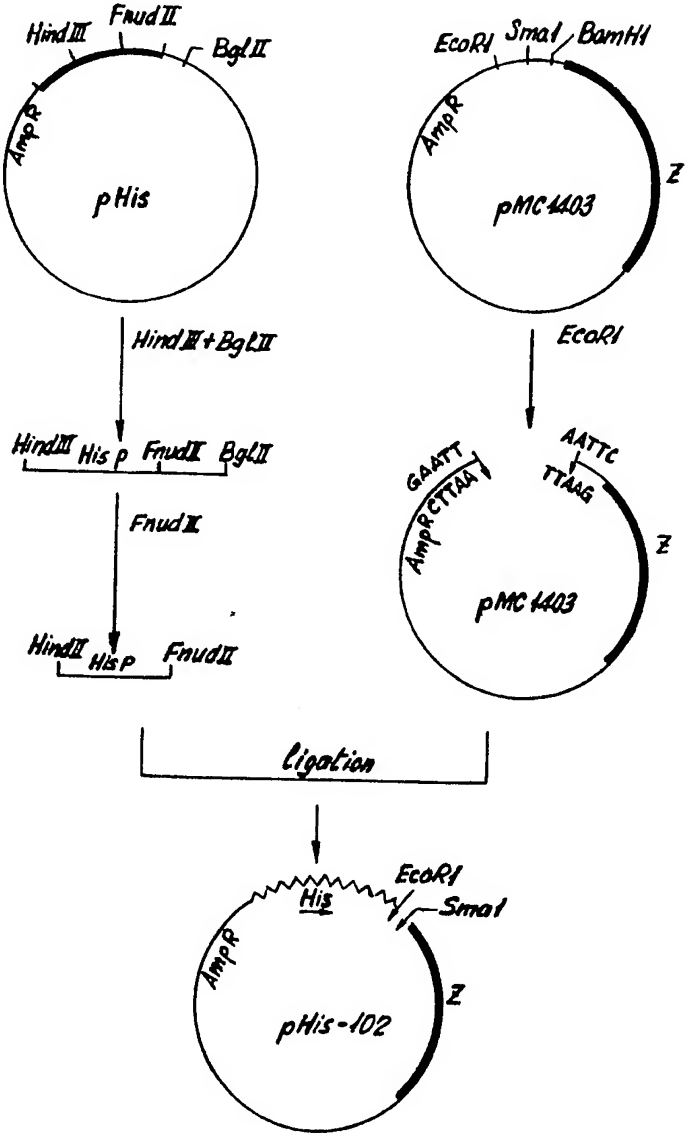

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FIG.10.

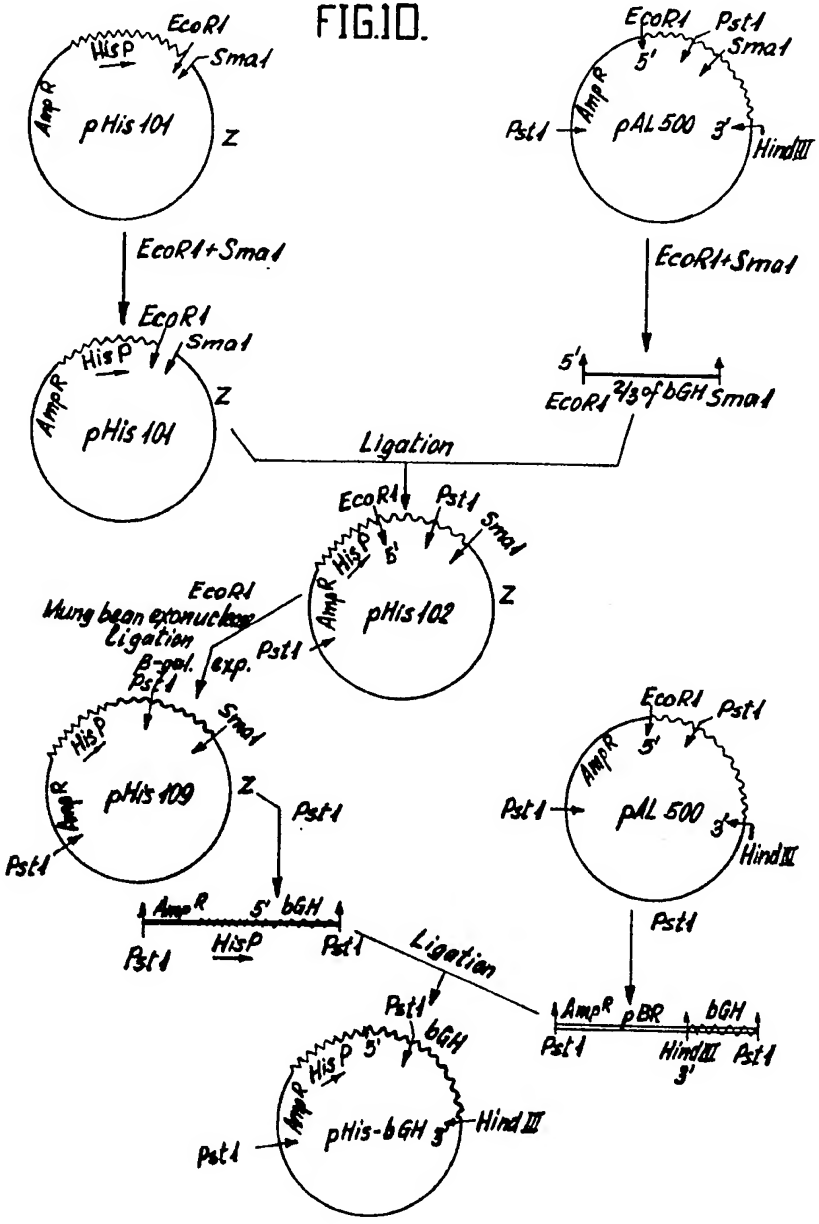

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FIG.11.

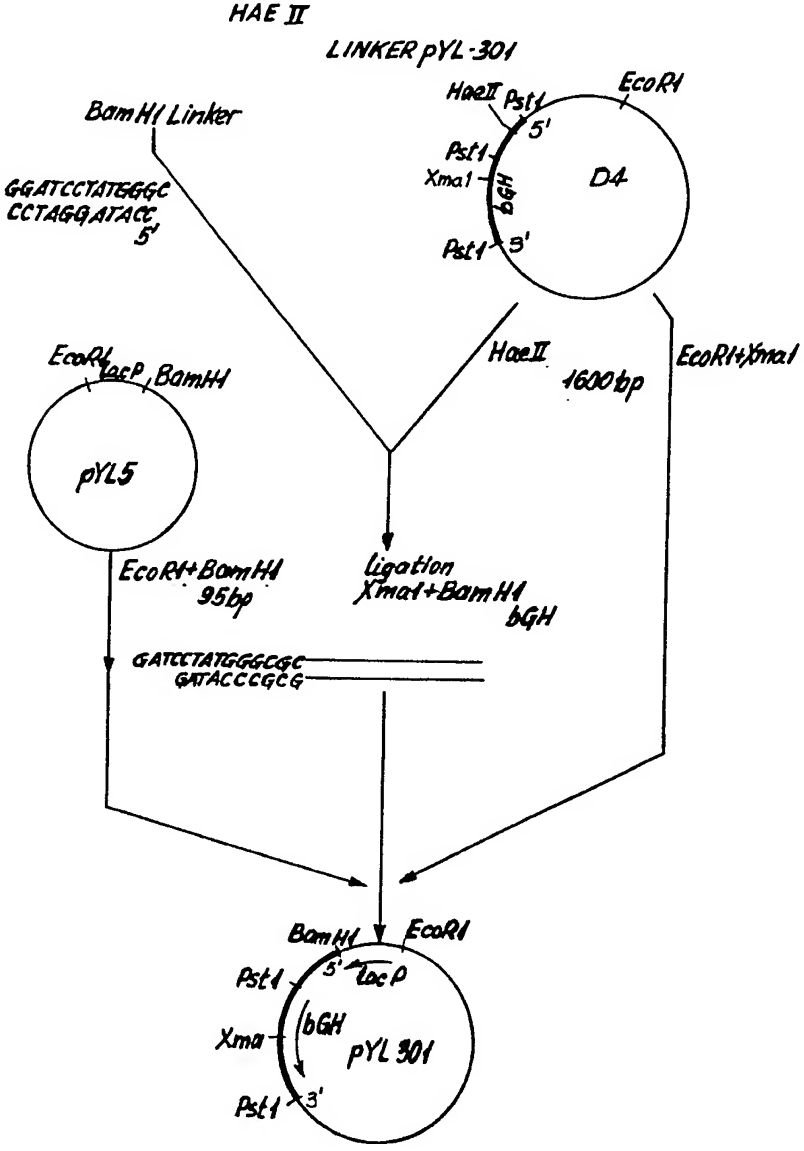

FIG.12.

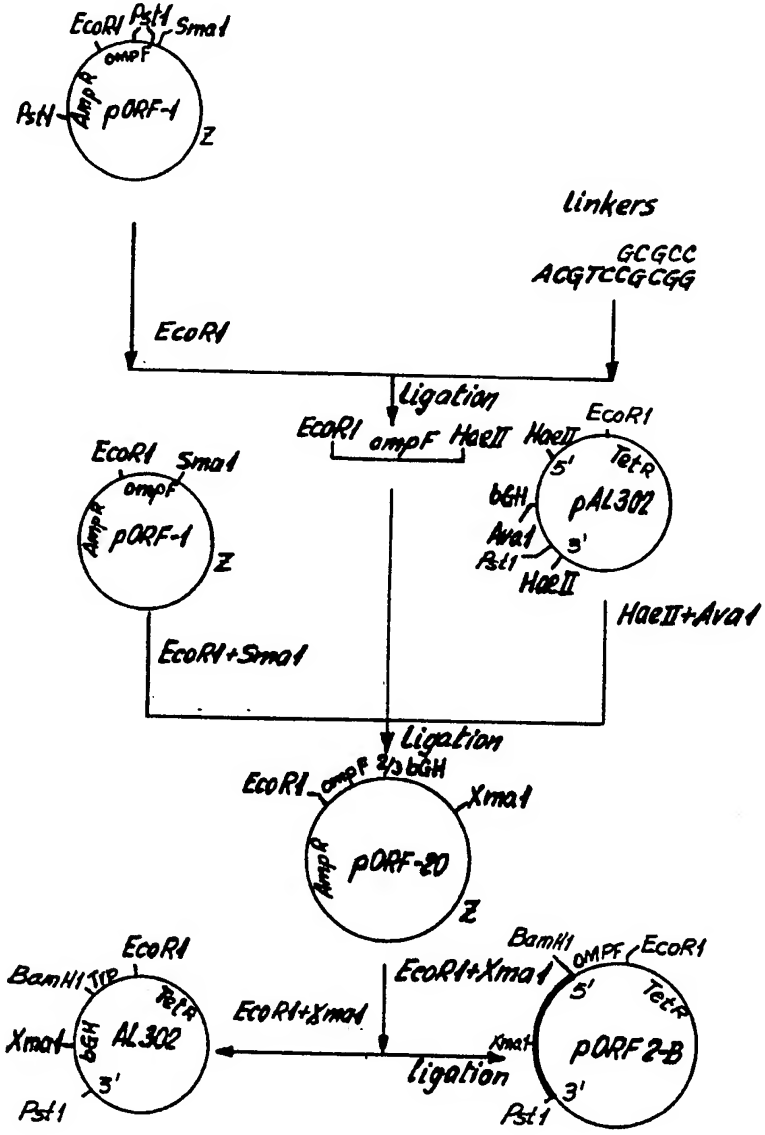
EcoR1
Pst1
Sma1
ompF
AmpR
pORF-1
Z
linkers
GCGCC
ACGTCCGCGG
EcoR1
Ligation
EcoR1
ompF
HaeII
HaeII
EcoR1
5'
TetR
bGH
pAL302
Ava1
3'
Pst1
HaeII
EcoR1
Sma1
ompF
AmpR
pORF-1
Z
HaeII+Ava1
EcoR1+Sma1
Ligation
EcoR1
ompF 2/3 bGH
Xma1
AmpR
pORF-20
Z
EcoR1
BamH1
TIP
TetR
Xma1
bGH
AL302
3'
Pst1
EcoR1+Xma1
EcoR1+Xma1
ligation
BamH1
OMPF
EcoR1
5'
TetR
Xma1
pORF2-B
3'
Pst1

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FIG.13.

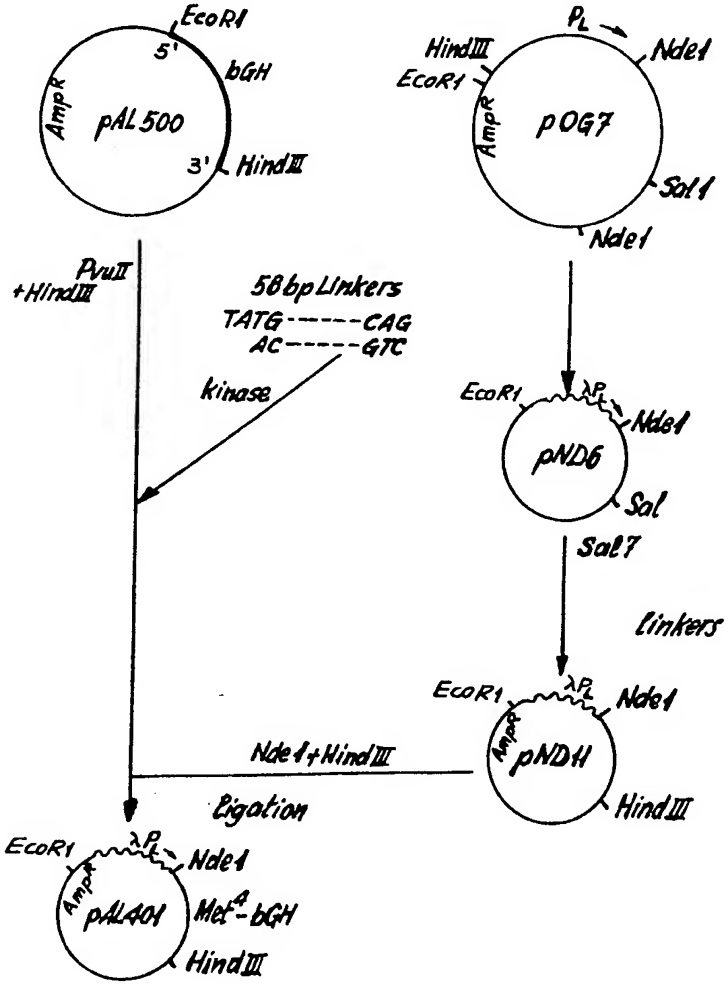


FIG.14.

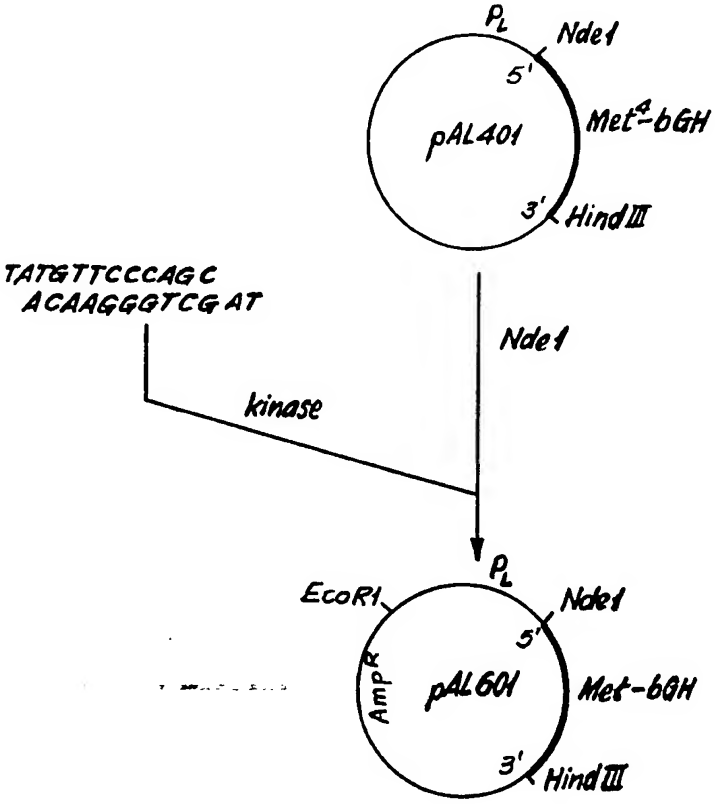
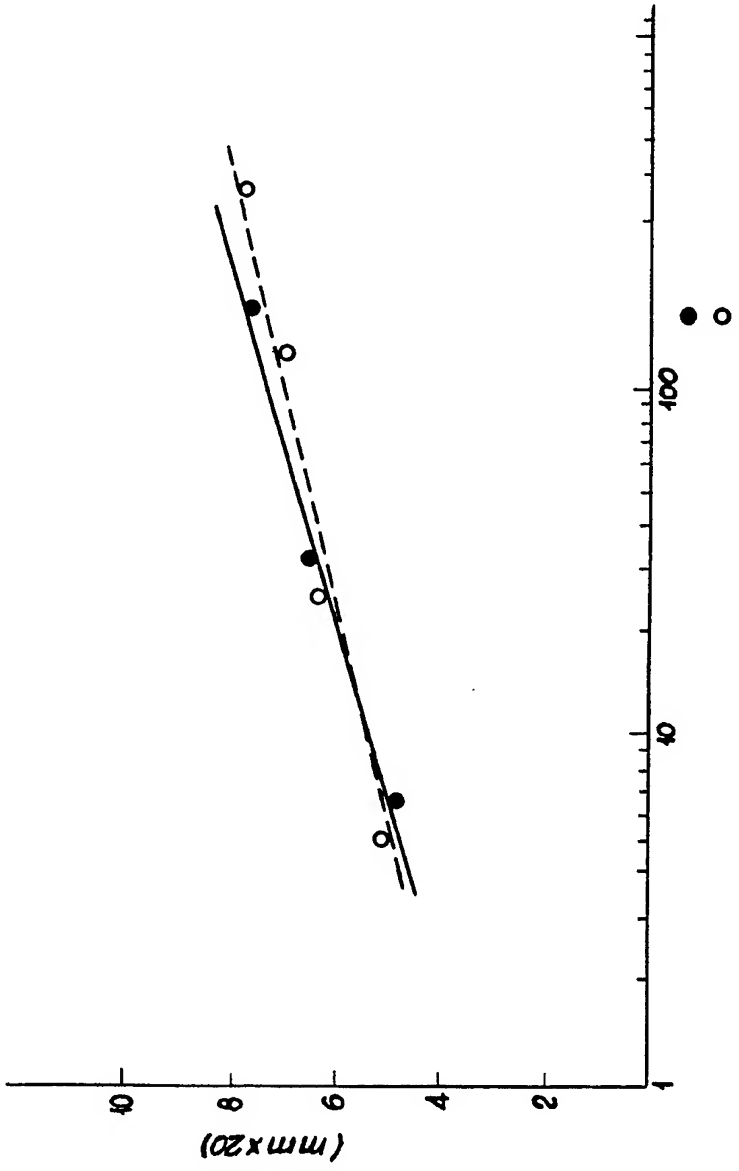

P_L
Nde1
5'
pAL401
Met^{-4}-bGH
3'
HindIII
TATGTTCCCAGC
ACAAGGGTCG AT
kinase
Nde1
P_L
EcoR1
Nde1
5'
Amp^R
pAL601
Met-bGH
3'
HindIII

FIG.15.



0131843
Application number

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

EP 84107717.5

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A,D	EP - A2 - 0 041 767 (BIOGEN N.V.) * Abstract; claims 1,3 * --	1,14	C 12 N 15/00 C 12 P 21/00 C 12 P 21/02 C 12 N 9/00 C 12 N 7/00 A 61 K 37/36 //C 12 R 1 19
A	EP - A2 - 0 080 848 (ELI LILLY AND COMPANY) * Claims 1,3,8,11 * --	1,2, 14-18	
A,D	GENE, vol. 5, no. 1, January 1979, Amsterdam H.U. BERNARD et al. "Construction of Plasmid Cloning Vehicles that Promote Gene Expression from the Bacteriophage Lambda p_L Promoter" pages 59-76 * Summary, page 59 * --	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N
C 12 P
A 61 K

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely: 1-63, 67-78
Claims searched incompletely: -
Claims not searched: 64-66
Reason for the limitation of the search:

Article 52(4) EPC

Place of search	Date of completion of the search	Examiner
VIENNA	18-10-1984	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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Application number

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

EP 84107717.5

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int Cl. 4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A,D	NATURE, vol. 292, no. 5819, July 9, 1981, New York, London H. SHIMATAKE et al. "Purified λ regulatory protein cII positively activates promoters for lysogenic development" pages 128-132 * Totality *	1,2	
A,D	DNA, vol. 2, no. 1, 1983, New York P.H. SEEBURG et al. "Efficient Bacterial Expression of Bovine and Porcine Growth Hormones" pages 37-45 * Totality *	1,14, 15,17, 43,44, 47,48, 68-70	TECHNICAL FIELDS SEARCHED (Int Cl. 4)
A,D	NATURE, vol. 281, no. 5732, October 18, 1979, London, New York D.V. GOEDDEL et al. "Direct expression in Escherichia coli of a DNA sequence coding for human growth hormone" pages 544-548 * Totality *	1,14, 16,45, 46,58-60,68, 72	

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 131 843 B1**

(12) # EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: **04.09.91**

(51) Int. Cl.5: **C12N 15/18**, C12P 21/00, C12P 21/02, C12N 9/00, C12N 7/00, A61K 37/36, //C12R1/19

(21) Application number: **84107717.5**

(22) Date of filing: **03.07.84**

Divisional application 88121246.8 filed on 03/07/84.

The file contains technical information submitted after the application was filed and not included in this specification

(54) **Expression vectors for enhanced production of polypeptides, plasmids containing the vectors, hosts containing the plasmids, products manufactured thereby and related methods.**

(30) Priority: **15.07.83 US 514188**

(43) Date of publication of application:
23.01.85 Bulletin 85/04

(45) Publication of the grant of the patent:
04.09.91 Bulletin 91/36

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI NL SE

(56) References cited:
EP-A- 0 041 767
EP-A- 0 080 848

GENE, vol. 5, no.1, January 1979, Amsterdam; H.U. BERNHARD et al.:"Construction of Plasmid Cloning Vehicles that Promote Gene Expression from the Bacteriophage Lambda pL Promoter", pp. 59-76

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EP 0 131 843 B1

NATURE, vol. 292, no. 5819, July 9, 1981, New York, London; H. SHIMATAKE et al.:"Purified Lamda regulatory protein cII positively activates promoters for lysogenic development", pp. 128-132

DNA, vol. 2, no. 1, 1983, New York; P.H. SEEBURG et al.:"Efficient Bacterial Expression of Bovine and Porcine Growth Hormones", pp. 37-45

NATURE, vol. 281, no. 5732, October 18, 1979, London, New York; D.V. GOEDDEL et al.:"Direct expression in Escherichia coli of a DNA sequence coding for human growth hormone", pp. 544-548

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Description

One aspect of genetic engineering involves the insertion of foreign DNA sequences derived from eukaryotic sources into Escherichia coli or other microorganisms. A further refinement of genetic engineering concerns inducing the resulting microorganism to produce polypeptides encoded by the foreign DNA. Production of polypeptides can be considered a two-step process, with each step including numerous substeps. The two steps are transcription and translation. To produce a polypeptide efficiently and in quantity both steps of the process must be efficient. Transcription is the production of mRNA from the gene (DNA). Translation is the production of polypeptide from the mRNA.

A critical substep of the transcription process is initiation, that is, the binding of RNA polymerase to a promoter-operator region. The sequence of deoxyribonucleotide bases which make up the promoter region may vary and thereby effect the relative efficiency of the promoter. The efficiency depends on the affinity of the RNA polymerase for the promoter.

The efficiency of translation is affected by the stability of the mRNA. Increased stability of the mRNA permits improved translation. Although the exact determinants of mRNA stability are not precisely known, it is known that mRNA secondary structure as determined by the sequence of its bases has a role in stability.

The initial substep of translation involves binding of the ribosome to a base sequence on the mRNA known as the Shine-Dalgarno sequence or the ribosomal binding site (RBS). The synthesis of polypeptides begins when the ribosome migrates along the mRNA to the AUG start codon for translation. Generally these codons are found approximately 10 bases "downstream" from the Shine-Dalgarno site. Factors which increase the efficiency of translation include those which enhance binding of the ribosomes to the Shine-Dalgarno site. It has been shown that the secondary structure of the mRNA in the region of the Shine-Dalgarno sequence and the AUG codon and the distance between the Shine-Dalgarno sequence and the AUG codon each play a critical role in determining the efficiency of translation. Other factors which affect the efficiency of translation are premature termination and attenuation. Efficiency of translation can be improved by removing the attenuation sites.

A difficulty encountered in attempts to produce high amounts of eukaryotic polypeptides in bacterial cells involves the inability of cells producing large amounts of mRNA to grow efficiently. This difficulty can be eliminated by preventing transcription by a process known as repression. In repression genes are switched off due to the action of a protein inhibitor (repressor protein) which prevents transcription by binding to the operator region. After microorganisms have grown to desired cell densities, the repressed genes are activated by destruction of the repressor or by addition of molecules known as inducers which overcome the effect of the repressor.

Numerous reports may be found in the literature concerning the cloning of eucaryotic genes in plasmids containing the P_L promoter from λ bacteriophage. (Bernard, H.V. et al., Gene (1979) 5, 59; Derom, C. et al., Gene (1982) 17, 45; Gheysen, D. et al., Gene (1982) 17. 55; Hedgpeth, J. et al., Mol. Gen. Genet. (1978) 163, 197. Remaut, E. et al., (1981) Gene 15, 81; and Derynck, R., et al., Nature (1980) 287, 193. In addition, European Patent Application No. 041.767, published December 16, 1981 describes expression vectors containing the P_L promoter from λ bacteriophage. However, none of these references describe the use of the C_{II} ribosomal binding site.

The use of a vector containing the P_L promoter from λ bacteriophage and the C_{II} ribosomal binding site has been described. (Oppenheim, A.B. et al, J. Mol. Biol. (1982) 158, 327 and Shimatake, H. and Rosenberg, M., Nature (1981) 292, 128.) These publications describe the production of increased levels of C_{II} protein but do not involve or describe the production of eucaryotic proteins.

In 1982 Shatzman and Rosenberg presented a poster at the 14th Miami Winter Symposium (Shatzman, A.R. and Rosenberg, M., 14 Miami Winter Symposium, abstract p98 [1982]). This abstract provides a non-enabling disclosure of the use of a vector containing P_L from λ bacteriophage, Nut and the C_{II} ribosomal binding site to synthesize a "eucaryotic" polypeptide (SV40 small T antigen is actually not a eucaryotic polypeptide but a viral protein) in an amount greater than 5% of the cell protein in an unnamed bacterial host. The operator used is not defined. Neither an origin of replication nor a gene for a selectable phenotype is identified. This system with which the vector is used is described as including certain host lysogens into which the vector can be stably transformed. The present invention in one embodiment, i.e., pMG100, may have certain similarities to this vector. However, it is not transformed into a host lysogen, but rather into suitable E. coli host strains which contain the thermolabile repressor C_I and the N gene but from which the rest of the lysogen has been removed. Moreover, it has been employed to produce bGH and hGH analogs in amounts in excess of 20% of total cell protein.

In addition, in other embodiments of this invention ribosomal binding sites which differ from C_{II} are employed. Also, in the presently most preferred vectors, pNDS and its derivatives, nonessential sequences

have been removed to create a vector permitting polypeptide production in amounts which are more than 10% greater than those obtained with pMG100.

Recently, applicants have learned of the existence of a pending U.S. patent application in the name of M. Rosenberg filed under Serial No. 457,352 by the National Institutes of Health, Dept. of Health and Human Services, U.S.A. Portions of this application have been obtained from the National Technical Information Service, U.S. Dept. of Commerce However, the claims are not available and are maintained in confidence. The available portions of the application have been reviewed. This disclosure is not enabling. It indicates that the host is important (p8, line 17) but fails to identify any suitable host. It further depends upon the use of a λ mutant which is not specified (p4, line 20). It indicates that the host contains lysogens (p8, line 18) unlike the present invention in which the host is not lysogenic. It mentions cloning and expression of a eucaryotic gene, monkey metallothionein gene, (p7, line 18) but does not provide details. It specifies that neither the sequence nor the position of any nucleotide in the C_{II} ribosomal binding region has been altered. (p3, line 27) In the present invention such alteration is possible.

No disclosure is present in the art concerning: successful expression with a P_L-C_{II} containing vector system of bovine or human growth hormones, production of bGH or hGH analogs having biological activity; compositions containing such analogs or uses of them; or induction methods for achieving polypeptide production in amounts greater than 20% of the total protein produced by the host.

The only disclosure in the art concerning production of bGH analogs by hosts transformed with genetically engineered vectors involves the use of the Trp promoter to produce a bGH analog having the amino acid Met at the N-terminus of the phenylalanine form of natural bGH (Seeburg, P.H. et al., DNA (1983) 2, 37.

The only disclosure in the art concerning production of hGH analogs by hosts transformed with genetically engineered vectors involves the use of the Lac and Trp promoters to produce an analog of hGH having the amino acid Met at the N-terminus of the natural hGH (Goedell, D.V. et al., Nature (1979) 281, 544)

SUMMARY OF THE INVENTION

This invention concerns a plasmid for the production of an animal growth hormone or polypeptide analog thereof having substantially the same amino acid sequence as, and the biological activity of, the naturally-occurring hormone in a suitable Escherichia coli host cell containing the thermolabile repressor C_I which plasmid renders the Escherichia coli host cell capable, upon increasing the temperature of the host cell to a temperature at which the repressor is inactivated, of effecting expression of DNA encoding the hormone or polypeptide analog thereof, wherein the plasmid comprises:

a double-stranded DNA molecule which comprises in 5' to 3' order the following:

the promoter and operator P_LO_L from λ bacteriophage;

an N utilization site for binding antiterminator N protein produced by the host cell;

a mutant C_{II} ribosomal binding site having the sequence

TAAGGAAGTACTTACAT

ATTCCTTCATGAATGTA;

an ATG initiation codon;

a DNA sequence encoding the hormone or polypeptide analog to be expressed in phase with the ATG initiation codon;

and which additionally includes a DNA sequence which contains an origin of replication from the bacterial plasmid pBR322 capable of autonomous replication in the Escherichia coli host cell and a gene associated with a selectable or identifiable phenotypic trait which is manifested when the plasmid is present in the Escherichia coli host cell.

Genes, i.e., cDNAs, encoding desired polypeptides such as animal growth hormones, e.g., bovine, porcine, chicken or human growth hormones, or analogs thereof may be inserted into the restriction enzyme site of the vector to create plasmids. The plasmids of the invention in turn can be introduced into suitable hosts where the genes can be expressed and the desired polypeptide produced. Preferred plasmids for bGH are pRec 2/3 and pROII; and for hGH, pTV 18(1) and pTV 104(2). Suitable hosts include Escherichia coli A1637, A1645, A2602 and A1563; A1637 being presently preferred.

The resulting host vector systems can be employed to manufacture polypeptides. The host cells containing the plasmids are grown under suitable conditions permitting production of polypeptide and the resulting polypeptide is recovered. Presently preferred conditions involve growth at about 42° C for 10 to 30 minutes, particularly 15 minutes, followed by continued growth at about 37-39° C for sufficient time to make the total growth period about 60-90 minutes, particularly growth at 38-39° C for about 75 minutes. Presently preferred growth media are lactalbumin hydrolysate with addition of glucose or brain heart infusion.

Using the above host-vector systems, analogs of bGH and hGH have been prepared. These analogs may be incorporated into veterinary or pharmaceutical compositions, respectively.

The respective analogs directly, or in such compositions, may be used to stimulate milk or meat production in a bovine or to treat human growth hormone deficiency.

DESCRIPTION OF THE FIGURES

FIG. 1. Construction of pMG100 expression vector. This plasmid was built by inserting a fragment of λ phage DNA contained between restriction sites HaeIII (location 38150) and Sau3a (location 38362) into a pKC30 plasmid DNA cleaved with HpaI and BamHI. The HaeIII-Sau3a fragment carries nut_R, t_{RI}, cy^- and ribosomal binding site of C_{II} protein (C_{II}-RBS). Subcloning of the C_{II}-RBS containing DNA into pKC30 creates pMG100 which contains a unique BamHI restriction site right after the ATG initiation codon of C_{II}-RBS and an NdeI restriction site within the ATG triplet (bottom inset). Numbers in parentheses denote location of restriction sites on the λ phage DNA.

FIG. 2. Construction of pRec 2/3 plasmid. A bGH cDNA containing plasmid, D_4, was digested with HaeII. A resulting 1600 bp large fragment was purified and subjected to digestion at 37° C for 5 minutes with 5 units of SI exonuclease. A synthetic EcoRI linker with the sequence:

```
GGAATTCC
CCTTAAGG
```

was attached by ligation The product was cleaved with EcoRI and inserted into pBR322 which had been cleaved with EcoRI. A clone, pALRI, was isolated which upon cleavage with EcoRI released a 1200 bp fragment with the sequence:

```
AATTCCCA....
    GGGT....
```

at the 5' end. Formation of this sequence demonstrates that pALRI contains an EcoRI restriction site which includes the TTC codon for residue number 1 (phenylalanine) of authentic bGH. pALRI was subjected to a partial cleavage with PstI. The digest was ligated with HindIII linkers and cleaved with EcoRI and HindIII. The fragment containing bGH cDNA was isolated and subcloned into pBR322 between EcoRI and HindIII restriction sites to give pAL500. The subcloned bGH cDNA fragment was then excised from pAL500 with EcoRI and HindIII, "filled in" with DNA polymerase "Klenow" fragment and inserted into the pMG100 expression vector (FIG. 1)opened at the BamHI site and also "filled in" as above. The resulting vector pREC 2/2, expresses a modified bGH which is altered at its amino terminus as follows:

$$MetAspGlnPhe^{1}Pro^{2}.....bGH$$

The plasmid pREC 2/2 was digested with PstI and the fragment containing the P_L promoter and the 5' end of the bGH gene (designated fragment A) was isolated. This fragment was ligated to a PstI fragment from pAL 500 (designated fragment B). The then resulting vector, pRec 2/3, expresses a modified bGH which is altered at its amino terminus as follows:

$MetAspGlnPhe^{1}Pro^{2}.....bGH$

FIG. 3. Construction of expression vectors pND5, pND55 and pROII. A plasmid pOG7 (A. Oppenheim, S. Gottesman and M. Gottesman, J. Mol. Biol. (1982) 158, 327) was cleaved with NdeI. The ends of the large fragment carrying the P_L promoter nut_L, t_R and C_{II}=RBS were ligated to give the pND5 expression vector. This pND5 vector DNA is opened with NdeI. Insertion of that NdeI fragment from pRec 2/3 (FIG. 2) which contains bGH cDNA results in a plasmid pROII which appears to be a better expressor of the modified bGH described in FIG. 2 than pRec 2/3. Insertion of synthetic linkers with the sequence:

TATGAGCTCA

ACTCGAGTAT

into pOG7 cleaved with NdeI results in an expression vector pND55 which contains a unique SacI restriction site in front of ATG. When pND55 is cleaved with SacI and treated with DNA polymerase "Klenow" fragment an ATG initiation codon results which follows the P_L promoter and C_{II}-RBS. This vector is suitable for expression of a wide variety of eukaryotic genes lacking an ATG initiation codon.

FIG. 4 Construction of pTV 18(1) and pTV 104(2). A plasmid, pTVHGH was prepared by cloning cDNA encoding hGH into the HindIII site of pBR 322 using standard methods. Meth. Enzymol. (1979) 68, 75. This plasmid was digested with HindIII. The resulting 800 base pair fragment was purified and further digested with FnuDII and "filled in" with DNA polymerase "Klenow" fragment. This treatment removes codons for the first 16 amino acids of hGH. The resulting DNA fragment is ligated with a synthetic linker which restores the codons for the sequence of hGH from Met^{14} and regenerates an NdeI restriction site in front of the ATG codon for Met^{14}. After treatment with NdeI this semi-synthetic DNA was inserted into the pND5 vector opened with NdeI. The resulting plasmid pTV 18(1) expresses hGH under control of the P_L promoter. This hGH is an analog missing the first 13 amino acid residues and having at its N-terminus Met^{14}.

Plasmid pTV 18(1) was partially digested with NdeI and ligated with a synthetic linker which contains the codons for amino acids 1-13 of hGH:

TATGTTCCCAACCATTCCATTATCCCGTCTGTTCGACAACGC

ACAAGGGTTGGTAAGGTAATAGGGCAGACAAGCTGTTGCGAT.

The linker is also complementary to the NdeI site on pTV 18(1) and positions the complete hGH gene in phase with the ATG initiation codon of the pND5 expression vector (FIG. 3). Thus, the resulting plasmid, pTV 104(2), expresses native hGH with an extra methionine at the N-terminus.

FIG. 5 shows the vector pAL Trp 46 which contains the Trp promoter and the first seven amino acids of the Trp E gene transcriptionally fused to the β-galactosidase gene.

FIGS. 6, 7 and 8 show a series of expression vectors (Tac) containing a part of the Trp promoter and Lac operator followed by restriction sites for insertion of a desired gene and expression of bGH under the control of Tac promoter.

FIGS. 9 and 10 show expression vectors containing bGH cDNA under the control of the histidine promoter.

FIG. 11 shows insertion of the bGH gene into an expression vector under the control of the Lac promoter.

FIG. 12 shows expression of bGH gene under control of Omp F promoter.

FIG. 13. Construction of Met^{4}-bGH analog pAL401 and expression vectors pND6 and pND11 with altered restriction sites:

pAL401 which expresses a modified form of bGH which is lacking the first three amino acids at the amino terminus of the bGH (Met^{4} bGH) was constructed by triple ligation of the following:

a) a bGH DNA fragment of 623 base pairs with PvuII and HindIII excised from pAL500

b) a linker formed by synthesizing two DNA strands which after purification were annealed to form:

CCATATGTCCTTGTCCGGCCTGTTTGCCAACGCTGTGCT

GCGACACGAGGCCCGAGTCGTGGACGTGGTCGACG

which was "filled in" with DNA polymerase "Klenow" fragment and then cleaved with NdeI and PvuII to prepare a 58 base pair fragment which was recovered and purified.

c) pND11 which was prepared as follows. An expression vector pOG7 was altered by elimination of HindIII and one of the NdeI sites (distant from the ATG initiator codon) to obtain pND6. Then HindIII linkers were introduced into a SalI site to give pND11.

FIG. 14. Construction of authentic bGH modified with methionine at the amino terminus and various analogs of bGH.

a) Plasmid pAL401 is treated with NdeI. A synthetic DNA linker containing an ATG initiation signal and the code for the first three amino acids at the amino terminus of native bGH is ligated into the NdeI site. The resulting vector pAL601 leads to the expression of native bGH containing an extra methionine residue at the amino terminus.

b) Using the strategy described in a) but modifying the structure of the oligodeoxyribonucleotide linker a class of vectors coding for a series of modified bovine growth hormones is constructed. The modified growth hormones start with methionine at the N-terminus and are followed by any of the twenty naturally occurring amino acids in each of positions 1 and 2, and any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp in position 3. Proceeding from position 4 to the COOH-terminus the sequence is identical to that of native bGH.

FIG. 15. Tibia test. This figure shows the comparison between effect of pRec 2/3 bGH analog and authentic bGH on the bone plate growth of hypophysectomized rats.

DETAILED DESCRIPTION OF THE INVENTION

The plasmid of the invention is derived from a vector which enables the achievement of enhanced levels of gene expression and polypeptide expression. The vector is a double-stranded DNA molecule. Upon introduction into a suitable bacterial host cell containing the thermolabile repressor C_I and increasing the temperature of the host to a temperature at which the repressor is destroyed, the vector renders the host cell capable of effecting expression of a desired gene inserted into the vector and production of polypeptide encoded by the gene.

The vector includes in 5' to 3' order the following:

a DNA sequence which contains the promoter and operator P_LO_L from lambda bacteriophage;

the N utilization site for binding antiterminator N protein produced by the host cell;

a DNA sequence which contains a ribosomal binding site for rendering the mRNA of the desired gene capable of binding to ribosomes within the host cell;

an ATG initiation codon or a DNA sequence which is converted into an ATG initiation codon upon insertion of the desired gene into the vector; and

a restriction enzyme site for inserting the desired gene into the vector in phase with the ATG initiation codon.

The vector also includes a DNA sequence which contains an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell and a DNA sequence which contains a gene associated with a selectable or identifiable phenotypic trait which is manifested when the vector is present in the host cell.

The host for use with the vector is Escherichia coli. The presently preferred strains are A1637, A1645, A2602 and A1563. A1637 is presently the most preferred strain. It was obtained from C600 by inserting transposon containing tetracycline resistance gene within the galactose operon as well as the lambda system for expression which is close to galactose operon. It has been deposited with the American Type Culture Collection in Rockville, Maryland, U.S.A. containing various plasmids as described more fully hereinafter All such deposits were made pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms.

A1645 was obtained from A1637 by selection for Gal+ (ability to ferment galactose) as well as loss of tetracycline resistance. It still contains the lambda expression system but part of the transposon has been removed by selection. Its phenotype is C600 r^-m^+ gal^+ thr^- leu^- lac Z^- (λcI857 Δ HI ΔBAM N+).

A2602 and A1563 are derived from SA500. Their phenotypes are SA500 his^-ilu^- gal+ Δ8-

(λCI857ΔHIΔBAM N+)and SA500 his⁻ ilu⁻gal+ Δ 8 lac ZxA21 (λCI859 int2 xisI nutL3 ΔHI), respectively.

Preferably the vector is a covalently closed circular double-stranded molecule. However, it is not essential that the vector be covalently closed.

The vector achieves its enhanced expression levels after the host cell is heated to a temperature at which the C_I repressor is destroyed. A temperature above about 42° C is effective for this purpose and since it is desired that unnecessary heat damage to the host cells be avoided to as great an extent as possible, it is generally desirable that the temperature never exceed 42° C by more than a few degrees.

One important component of the vector is the ribosomal binding site which is a synthetic oligonucleotide having the sequence:

TAAGGAAGTACTTACAT

ATTCCTTCATGAATGTA

Another component of the vector is the restriction enzyme site for insertion of desired genes into the vector in phase with the ATG initiation codon. Numerous such sites may be used. The presently preferred sites are BamHI, SacI and NdeI.

The vector also includes an origin of replication from a bacterial plasmid capable of autonomous replication in the host cell which is derived from pBR322.

A DNA sequence which contains a gene associated with a selectable or identifiable phenotypic trait which is manifested when the vector is present in the host cell is also a component of the vector. Suitable genes include those associated with temperature sensitivity or drug resistance, e.g., resistance to ampicillin, chloramphenicol or tetracycline.

Relative to vectors previously described in the scientific literature, the present vectors are used to obtain enhanced expression of genes encoding animal growth hormones, e.g., bovine, porcine, chicken or human growth hormones or analogs of any of the preceding. By analog is meant a polypeptide having the same activity as the naturally occurring polypeptide but having one or more different amino acids at the N-terminus of the polypeptide.

The vector may be formed by methods well known to those skilled in the art to which the invention relates. Such methods are described in greater detail in various publications identified herein, the contents of which are hereby incorporated by reference into the present disclosure in order to provide complete information concerning the state of the art.

One presently preferred vector is pMG100 having the restriction map shown in FIG. 1. This vector has had cDNA encoding bovine growth hormone inserted into its BamHI restriction site. The resulting plasmid is designated pRec 2/3. Its restriction map is shown in FIG. 2. The plasmid pRec 2/3 was introduced into Escherichia coli strain A1637 using conventional transformation methods. The resulting host vector system has been deposited under ATCC No. 39385.

A second presently preferred vector is pND5 having the restriction map shown in FIG. 3. Bovine growth hormone cDNA has been inserted into its NdeI restriction site. The resulting plasmid is designated pROII. Its restriction map is also shown in FIG. 3. The plasmid pROII was introduced into E. coli strain A1637 via transformation. The host vector system which resulted has been deposited under ATCC No. 39390.

The vector pND5 has also been used to clone human growth hormone. One plasmid designated pTV 18(1) and another designated pTV 104(2) have been created by inserting hGH cDNA into the NdeI restriction site. pTV 18(1) is shown in FIG. 4. It has been introduced into E. coli strain A1637 via transformation. The resulting host vector system has been deposited under ATCC No. 39386. pTV 104(2) is shown in FIG. 4. It also has been introduced into E. coli strain A1637. The resulting host vector system has been deposited under ATCC No. 39384.

Using the same approach other plasmids may be prepared by inserting into the restriction enzyme site of the present vector a gene encoding the desired polypeptides.

The preceding specific host vector systems involve E. coli A1637. However, as previously indicated other strains have been used including A1645, A2602 and A1563. These host vector systems may be used to produce polypeptides such as bovine and human growth hormones. To do so the host vector system is grown under suitable conditions permitting production of the polypeptide which is then recovered.

Suitable conditions involve growth of the host vector system for an appropriate period of time at about 42° C followed by continued growth at about 37-39° C for an additional period of time, the growth being carried out on a suitable medium.

Desirably the initial period of growth is about 10 to 30 minutes at 42° C followed by growth at 37-39° C for a sufficient period of time such that the total period of growth is about 60 to 90 minutes. Preferably the growth is for about 15 minutes at 42° C followed by about 75 minutes at 38-39° C. Suitable media include lactalbumin hydrolysate with addition of glucose or brain heart infusion. In order to stably maintain the vector in the host it is critical that the host be maintained under selective pressure, e.g., addition of antibiotic.

By means of the preceding method a number of bGH and hGH analogs has been prepared. These have or may have the activity of the naturally occurring hormones.

bGH analogs have the activity of natural bGH and an identical amino acid sequence except for variations at the N-terminus of up to five (5) amino acids. Examples include the following:

1) amino acid methionine added to N-terminus of the phenylalanine form of bGH.
2) amino acid methionine added to N-terminus of the alanine form of bGH.
3) amino acid sequence Met-Asp-Gln added to N-terminus of the phenylalanine form of bGH.
4) amino acid sequence Ala-Gly added to N-terminus of the alanine form of bGH.
5) amino acid sequence Met-Gly added to N-terminus of the alanine form of bGH.
6) amino acid sequence Met-Asp-Pro-Met-Gly added to N-terminus of the alanine form of bGH.
7) amino acid sequence Met-Asp-Pro added to N-terminus of the phenylalanine form of bGH.
8) amino acid sequence Met-Thr-Arg added to N-terminus of the phenylalanine form of bGH.
9) amino acids up to methionine (4 position) removed from N-terminus of phenylalanine form of bGH.

An analog of bGH having the amino acid sequence:

Met-$(X)_n$-Y-Met...

wherein Met is the N-terminus, X is any of the twenty naturally occurring amino acids, Y is any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp, n is an integer from 0 to 6 and Met... is the sequence of natural bGH from position 4 to the COOH-terminus (position 191).

hGH analogs have the activity of natural hGH and an identical amino acid sequence except for variations at the N-terminus. Examples include the following.

1) amino acid methionine added to N-terminus of natural hGH.
2) amino acids up to methionine (14 position) removed from N-terminus of hGH.

An analog of hGH having the amino acid sequence:

Met-$(X)_n$-Y-Met...

wherein Met is the N-terminus, X is any of the twenty naturally occurring amino acids, Y is any of the twenty amino acids other than Glu, Gln, Lys, Met or Trp, n is an integer from 0 to 13 and Met... is the sequence of natural hGH from position 14 to the COOH-terminus (position 191).

Veterinary compositions may be prepared which contain effective amounts of one or more bGH analog and a suitable carrier. Such carriers are well-known to those skilled in the art. The analogs may be administered directly or in the form of a composition to a bovine in order to increase milk or meat production.

Pharmaceutical compositions may be prepared which contain effective amounts of one or more hGH analog and a suitable carrier. Such carriers are well-known to those skilled in the art. The analogs may be administered directly or in the form of a composition to a human subject, e.g., one afflicted by dwarfism, to treat deficiencies in hGH production by the subject.

EXAMPLES

The examples which follow are set forth to aid in understanding the invention but are not intended to, and should not be so construed as to, limit its scope in any way. The examples do not include detailed descriptions for conventional methods employed in the construction of vectors, the insertion of genes encoding polypeptides of interest into such vectors or the introduction of the resulting plasmids into bacterial hosts. Such methods are well-known to those skilled in the art and are described in numerous publications including the following:

Principles of Gene Manipulation, An Introduction to Genetic Engineering, 2nd Edition, edited by R.W. Old and S.B. Primrose, Univ. of Calif. Press (1981)

Met. Enzymol. vol. 68, Recombinant DNA, edited by Ray Wu (Academic Press 1979)

Met. Enzymol. vol. 65, Nucleic Acids (Part 1), edited by Lawrence Grossman and Kivie Moldave (Academic Press 1980)

T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory, NY (1982)

H.V. Bernard et al., Gene (1979) 5, 59

A.B. Oppenheim et al., J. Mol. Biol. (1982) 158, 327
E. Remaut et al., Gene (1981) 15, 81

EXAMPLE 1

EXPRESSION VECTORS

As used herein the term expression vector refers to a group of plasmids useful for expressing desired genes in bacteria, particularly in E. coli. The desired gene may be inserted into the expression vector or alternatively, the promoters on the expression vector may be excised and placed in front of the desired gene.

I. P_L EXPRESSION VECTORS

A. pMG 100

pMG 100, as shown in FIG. 1 and described in detail under Description of the Figures is composed of λ DNA inserted into the multicopy plasmid pBR322. The salient features of the λ DNA is that it contains the λ P_L promoter, N utilization sites L and R (nut_L and nut_R) termination R1 site (t_{RI}), the C_{II} ribosomal binding site and an ATG initiation codon. Other features are shown in FIG. 1.

pMG100 was prepared from pKC30. pKC30 in turn was prepared by subcloning of λP_L promoter in the following manner.

λ phage DNA was digested with XhoI and SmaI restriction endonucleases and the unique fragment comprised of 6393 base pairs was purified and subsequently digested with HindIII and BamHI restriction endonucleases. The resulting fragment comprised of 2397 base pairs and containing P_L promoter was purified and ligated into a pBR322 DNA large fragment isolated from the HindIII and BamHI digest. The subclone was identified by colony hybridization, recovered and plasmid DNA isolated (Oppenheim, A. et al., J.Mol.Biol. (1982) 158, 327).

This plasmid and its derivatives containing eukaryotic genes may be maintained in suitable E. coli hosts. The most important feature of the host is that it provides the thermosensitive repressor CI857 and the antitermination N protein (Gottesman, M.E. et al., J.Mol.Biol. (1978) 140, 197).

This vector has numerous advantages over previously described expression vectors including:

1. Extremely High Levels of Expression

This vector is capable of directing expression of foreign proteins in E. coli at levels as high as 15-25% of the total cellular protein.

2. Thermoinducible Regulation of Expression

The P_L promoter is inactive when the CI repressor is bound to it. The CI857 repressor is thermosensitive, that is, it binds to the promoter at 30° C but is inactived at 42° C. Thus, by increasing the temperature of fermentation to 42° C the host bacteria are induced to produce the desired protein.

The advantages of such a system include the following:

(a) a foreign protein which is toxic to E. coli can be produced when desired thus avoiding cell death early in the fermentation process.

(b) overproduction of a protein may stabilize it and prevent proteolytic degradation. (Cheng, Y.E. et al., Gene (1981) 14, 121) Thus, "instantaneous" overproduction using a tightly regulated promoter such as P_L may be preferable to continuous low level production.

3. High Copy Number

The P_L promoter in pMG100 is found on a plasmid with a high copy number in distinction to λ itself which is present in low copy numbers in E. coli. This increases expression levels.

4. Ribosome Binding Site and Initiation Codon

This expression vector contains a strong procaryotic ribosomal binding site (RBS) as well as a translation initiation codon (ATG) Thus, any eukaryotic gene may be cloned without the need for adding an initiation codon. Furthermore, the efficient RBS increases levels of expression.

5. Convenient Restriction Site

The expression vector has a BamHI site located directly following the ATG initiation codon which permits proper positioning of the desired gene in order to achieve optimal expression.

6. Nut Site

N protein which is provided by the host binds to Nut site on the expression vector and thereby prevents termination of transcription at the t_{RI} site.

B. pND5

As shown in FIG. 3, pND5 contains the P_L promoter and the other important components of the expression vectors of this invention. It includes a unique NdeI site immediately after the ribosomal binding site. The ribosomal binding site differs from the normal C_{II} site. It has the sequence:

TAAGGAAGTACTTACAT

ATTCCTTCATGAATGTA

It may be derived from a mutant or may be chemically synthesized. As described in detail under Description of the Figures pND5 was derived from pOG7. (Oppenheim, A., et al., J.Mol.Biol. (1982) 158, 327) This vector does not contain a translation initiation codon. It appears to provide superior expression of modified bGH and hGH, particularly enhanced yield relative to pMG100 containing a bGH analog.

C. pND55

pND55 is a derivative of pND5 which contains the convenient restriction site SacI in front of C_{II}-RBS and ATG initiation codon. Cleavage of the plasmid at this site and subsequent treatment with DNA polymerase Klenow fragment allows one to obtain an ATG initiation codon to which any desired gene can be ligated. (FIG. 3 and Description of FIG. 3.)

II. TRP EXPRESSION VECTORS

A. pAL Trp 46

pAL Trp 46 contains the Trp promoter and the first seven amino acids of the Trp E gene fused to the β- galactosidase gene. (FIG. 5). The desired gene can be inserted into a BamHI site which follows the 7 amino acids of Trp E.

B. pAL Trp 47; Trp 46 Deleted of Attenuator

This is a construction based on Trp 46 in which the attenuator region of the Trp promoter has been deleted.

C. Trp-Lac Fusions

The construction of this promoter, found on plasmid p4754 is illustrated in FIGS. 6 and 7. A variation of this construction is outlined in FIG. 8.

III. Histidine Promoter Expression Vectors

The construction of this expression vector is illustrated in FIGS. 9 and 10.

IV. Other Promoters Used

A. Lac

This promoter was used in the construction of pYL 301 as shown in FIG. 11.

B. Omp F

This is a promoter system which expresses a protein attached to a signal sequence. The signal sequence is removed when the protein is translocated across the membrane. (FIG. 12)

EXAMPLE 2

Bovine Growth Hormone

The starting point for bGH cDNA modifications is plasmid D_4 which has been described previously. (Keshet, E. et al, Nucleic Acids Research (1981) 9, 19). The D_4 plasmid is also described in pending U.S. patent application, Serial No. 245,943, filed March 20, 1981, claiming priority of Israel patent application, Serial No. 59,690 filed March 24, 1980. It has previously been deposited with the American Type Culture Collection in an E. coli host under ATCC No. 31826.

I. pRec 2/3 bGH

The construction of pRec 2/3 is shown in FIG. 2 and described in the Description of the Figures. bGH cDNA from D_4 has been manipulated prior to insertion into PMG100 to provide the correct reading frame.

pRec 2/3 has been introduced into various E. coli strains including A1637 by transformation using known methods. A1637 containing pRec 2/3 has been deposited under ATCC No. 39385. This strain produces upon growth and induction an analog of bGH having the amino acid sequence Met-Asp-Gln added to the N-terminus of the phenylalanine form of natural bGH. The amount of bGH analog produced by pRec 2/3 was about 23% of the total protein produced by the bacteria as calculated from scanning of Coomasie stained SDS polyacrylamide gels.

II. pROII

The construction of pROII is shown in FIG. 3 and described in the Description of the Figures. The pND5 vector DNA is restricted with NdeI. Insertion of the NdeI fragment from pRec 2/3 (FIG. 2) which contains bGH cDNA results in the plasmid pROII.

pROII has been introduced into E. coli A1637 by transformation. The resulting host vector system has been deposited under ATCC No. 39390. This strain when grown and induced produces the same analog as pRec 2/3. Preliminary results indicate that pROII produces up to 20% more bGH analog than pRec 2/3. The methods used to grow the strain, recover the bGH analog produced and purify it are the same as those described for pRec 2/3 in Example 4.

III. pAL401

The construction of pAL401 is shown in FIG. 13 and described in the Description of the Figures bGH cDNA from D_4 by way of pAL-500 (FIG. 2) was inserted into pND11 as shown in FIG. 13.

pAL401 may be introduced into E. coli A1637 by transformation. The resulting strain produces an analog of bGH in which Met^4 of natural bGH is at the N-terminus and the amino acids preceding Met^4 have been deleted.

IV. pAL601

The construction of pAL601 is shown in FIG. 14 and described in the Description of the Figures. It is a derivative of pAL401 (FIG. 13).

pAL601 may be introduced into E. coli A1637 by transformation. The resulting strain produces an analog of bGH in which Met has been added to the N-terminus of the phenylalanine form of bGH.

EXAMPLE 3

Human Growth Hormone

The starting point for hGH cDNA was cloning of the cDNA from mRNA purified from hypophyses tumor from acromegalic patients into the HindIII site of pBR322.

I. pTV 18(1)

The construction of PTV 18(1) is shown in FIG. 4 and described in the Description of the Figures. hGH cDNA was manipulated prior to insertion into pND5 to provide the correct reading frame.

pTV 18(1) was introduced into E. coli A1637 by transformation. The resulting bacteria have been deposited under ATCC No. 39386. This strain upon growth and induction produces an analog of hGH having

the sequence of natural hGH beginning with Met^{14} and lacking amino acids 1-13. The amount of hGH analog produced by pTV 18(1) was about 8% of the total protein produced by the bacteria.

II. pTV 104(2)

The construction of pTV 104(2) is shown in FIG. 4 and described in the Description of the Figures. hGH cDNA was manipulated prior to insertion into pND5 to provide the correct reading frame.

pTV 104(2) was introduced into E. coli A1637 by transformation. The resulting bacteria have been deposited under ATCC No. 39384. This strain upon growth and induction produces an analog of hGH having the sequence of natural hGH preceded by Met at the N-terminus. The amount of hGH analog produced by pTV 104(2) was above 25% of the total protein produced by the bacteria.

EXAMPLE 4

Growth of pRec 2/3

Stock Cultures: Stock cultures of pRec 2/3 in A1637 are grown on BHI medium (see inoculum), then diluted twofold with 87% glycerol containing phosphate citrate buffer, and stored at -70° C.

Inoculum: Inoculum is propagated in BHI medium (37 g/l) brain heart infusion (DIFCO). Sterile medium in shake flask is inoculated from stock culture and incubated 15 hours on shaker at 30° C, 200 r.p.m. Subsequent stages in inoculum propagation are carried out in stirred aerated fermentors. Sterile medium is inoculated with 0.2 ml flask culture per l, and incubated 15 hours at 30° C, pH 7± 0.5 with agitation and aeration to maintain dissolved oxygen level above 20% air saturation.

Production: Production medium contains:

Lactalbumin hydrolysate (enzymatic)	20 g/l
Yeast extract	10 g/l
K_2HPO_4	2.5 g/l
NaCl	10 g/l
Ampicillin	0.1 g/l
Biotin	0.1 mg/l
Thiamine	1 mg/l
Trace elements solution	3 ml/l

Ampicillin, biotin and thiamine in solution are filter sterilized separately and added to the sterile production medium before inoculation. Sterile glucose solution is added initially to supply 10 g/l, and during the induction and expression procedure to maintain glucose above 10 g/l

Trace elements solution contains:

$MgSO_4 \cdot 7H_2O$	170 g/l
$FeCl_3$	16 g/l
$ZnCl_2 \cdot 4H_2O$	2 g/l
$CoCl_2 \cdot 6H_2O$	2 g/l
$Na_2MoO_4 \cdot 2H_2O$	2 g/l
$CaCl_2 \cdot 2H_2O$	1 g/l
$CuCl_2$	1 g/l
H_3BO_3	0.5 g/l
Conc. HCl	100 ml/l

The medium is inoculated with 5-10% inoculum culture and incubated at 30° C. Agitation-aeration rates are set to maintain dissolved oxygen level above 20% air saturation. The pH is maintained at 7±0.2 with NH_3. Once cell concentration reaches about 3 g/l (OD_{660} = 10) induction is started.

Temperature is raised to 42° C. Maintained there for 15 minutes, then lowered to 38° C. Following incubation at 38° C for 1- 1 1/2 hours, the culture is chilled, and cells are recovered by centrifugation for hormone purification.

Recovery of bGH

One kilogram of bacterial cells is suspended in 10 volumes of the solution containing 50 mM Tris-Cl (pH 7.4), 50 mM EDTA and 25% sucrose in a Warring blender, with a control of blender's speed to minimize foaming. The homogeneous suspension is continuously passed through a Dynomill cell disruptor (Willy A. Bachofen, Basel) and the homogeneous suspension of disrupted cells is clarified first by centrifugation in a Sharpless centrifuge followed by a continuous centrifugation at 20,000 rpm in a Sorvall centrifuge. The precipitate from both centrifugation steps is collected, washed with 50 mM Tris-Cl (pH 7.4) and resuspended in 500 ml of the same buffer. Lysozyme is added to a final concentration of 2 mg/ml and the suspension is incubated for 1 hour at 37° C. Triton X-100 is then added to a final concentration of 1%, the suspension is cooled to 4° C and centrifuged at 20,000 rpm for 20 minutes in a Sorvall SS34 rotor. The precipitate is collected, washed twice with 50 mM Tris-Cl, resuspended in 500 ml of 50 mM Tris-Cl (pH 7.4),5 mM $MgCl_2$ and deoxyribonuclease is added to a final concentration of 20 µg/ml. After incubation for 30 minutes at room temperature the precipitate is collected as above, washed twice with 500 ml of 20 mM Tris-Cl (pH 7.4), 100 mM NaCl and 10 mM EDTA, followed by two washings with 500 ml of distilled water. The precipitate is collected by centrifugation and can be stored at -20° C for an indefinite time. At this stage the bGH is 80% pure as judged by sodium dodecyl sulfate-gel electrophoresis. The yield is approximately 15 g of bGH.

Purification of bGH

One hundred gr of precipitate is suspended in 40 ml distilled water and solubilized by titration with 0.5 M NaOH, PH 11.8. The solution is then sonicated for 2 minutes and clarified by centrifugation at 20,000 rpm in a Sorvall SS34 rotor for 20 minutes. The solution is then applied onto a Sepharose CL-6B column (5 x 100 cm) equilibrated with 6.5 mM borate buffer, pH 11.8. Column is developed at the rate of 100 ml/hr and fractions of 12 ml are collected. The first peak off the column is discarded. The following two peaks are separated and pooled. The first represents aggregated bGH with low activity; the second bGH with high activity.

A DEAE-Sephacel (25 g/100 gr. equiv. ppt) column is equilibrated with 6.5 mM borate buffer, pH 9.0. The second bGH peak is brought to pH 9.0 with HCl loaded on the DEAE Sephacel column at a rate of 250 ml/hr. The column is washed with 7.5 ml of 6.5 mM borate buffer, pH 9.0, eluted with 6.5 mM borate buffer, pH 9.0 containing 75 mM NaCl. The fractions with OD_{280} above 0.3 are pooled, dialysed against H_2O in Millipore Pellicon dialysis apparatus and then lyophilised.

EXAMPLE 5

Activity of bGH Analog Produced by pRec 2/3

1. Radioimmunoassay comparison of bGH analog with natural bGH

A solution containing 100 ng/ml bGH analog was prepared in phosphate buffered saline (1% BSA). This solution was diluted serially to concentrations of 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 ng/l. Duplicate 0.1 ml aliquots of these solutions were submitted to RIA using a double antibody procedure. The dilution curve was comparable to that obtained with natural bGH.

2. Radioreceptor binding Assay

A radioreceptor binding assay was performed with rabbit liver membranes as described by T. Tushima and H.G. Freisen (Y. Chin., Endocr. Metab. (1973) 37, 334 using ^{125}I-hGH as the tracer and authentic bGH solutions for the construction of calibration curves. Samples were incubated in triplicate for two hours at room temperature in 0.3 ml of assay buffer (50 mM Tris, 15 mM $CaCl_2$ and 5 mg/ml bovine serum albumin, pH 7.6). The tubes contained ^{125}I-hGH (20,000 cpm of preparation of 30-60 μci/ μg), 150-250 μg liver membrane protein and either natural bGH (1-100 ng) or extracts of bacterial bGH. The result demonstrated that the bGH activity of the bGH analog is comparable to that of natural bGH.

3. Tibia Test

The bioactivity of the pRec 2/3 bGH analog recovered from engineering bacterial cells according to Example 4 was evaluated by a tibia test. (Parlow, A.F., et al., Endocrinology (1965) 77, 1126.)

Rats were hypophysectomized at 28-30 days of age, then kept for 10-14 days without treatment. Bovine growth hormone derived from bovine pituitaries or from recombinant E. coli was dissolved in 0.15M NaCl + 0.01 M borate, pH 10.0. Rats (4-7 per group) received daily subcutaneous injections of bGH solutions (5-125 μg/day in 0.2 cc) for 5 days while kept on a normal diet (Purina Rat-Chow and water ad-libitum). The animals were sacrificed on the 6th day, their foreleg knee-bones taken out, cut longitudinally, fixed with acetone and stained with 2% $AgNO_3$. The width of the epiphyseal plates were measured by observation through a dissecting binocular (Nikon). Mean values (of 40 readings per rat) were used for the construction of log dose-response curves. Results are shown in FIG. 15.

EXAMPLE 6

bGH Analogs

Table I sets forth a series of plasmids which have been constructed and the analogs which were produced from them.

TABLE I

PLASMID	AMINO TERMINUS OF bGH ANALOGS
Rec 2/3	Met Asp Gln Phe[2]
pB 1	Met Asp Pro Met Gly Ala Phe[2]
pM 4	Met Asp Pro Phe[2]
pM 1	Met Ala[1] Phe[2]
pM 2	Met Ala[1] Phe[2]
pAL 401	Met[4]
pYL 301	Met Gly Ala[1] Phe[2]
pAL 302	11 A.A + Ala[1] Phe[2]
pHis 129	Met Thr Arg Phe[2]
pAL 312	Met Gly Ala[1] Phe[2]
pAL 322	Met Gly Ala[1] Phe[2]
pAL 601R	Met Gly Ala[1] Phe[2]
p 18	Met Gly Ala[1] Phe[2]
PBTG-800	Met Glu Phe[2]
pORF 2-12	Ala Gly Ala[1] Phe[2]

EXAMPLE 7

Effect of pRec 2/3 bGH analog on Lactogenesis in Dairy Cows

The lactogenic effect of bGH has been well documented in the scientific literature in the reports of Bines, J. et al, Brit J. Nutri. (1980) 43, 179 and Peel, C. et al, J. Nutr. (1981) 111, 1662. Bauman, D. et al, J. Dairy Sci. Vol. Supp. 1, Abst 86 (1982) reported that milk production was increased by rDNA bGH. An experiment was conducted to determine the effects of pRec 2/3 bGH on lactogenesis in comparison with natural bGH. Eighteen Holstein cows ranging from 141 to 154 days postpartum were randomly assigned to treatment and blocked according to milk production according to the following design.

Pretreatment	Treatment	Daily GH Injection
Control	5 days	Saline
Natural bGH	5 days	25 mg/day for 10 days
pRec 2/3 bGH	5 days	25 mg/day for 10 days

The bGHs were put in solution with 0.1 M $NaHCO_3$ aqueous buffer (pH = 8.2) at the concentration of 1

mg/ml immediately prior to each day's injections. The cows were injected with placebo or bGH solution daily for 10 days in a subcutaneous site in the neck region. No injections were given during the 5-day pretreatment period.

The cows were milked twice daily at approximately 6:00 a.m. and 5:00 p.m. Milk weights were recorded by the Boumatic system and recorded in the dairy data system.

The average milk production values for the pretreatment and bGH treatment periods are shown in Table II. The production level of the control cows was unchanged while the milk volume increased to a similar degree in both the bGH groups. The natural bGH caused an 11.9% increase in milk for a 10-day period and bGH analog treatment resulted in a 10.2% increase. The data were not analyzed for statistical significance due to the small number of animals, however, the magnitudes of the increases are similar to those reported in the literature.

It was concluded that pRec 2/3 bGH stimulates lactogenesis in dairy cows similar to natural bGH.

TABLE II

Bovine Growth Hormone Effect on Lactogenesis
Natural bGH vs pRec 2/3 bGH

Treatment Group	No.	Av. Daily Milk Production lb/day		
		Pretreatment 5 days	During GH 10 days	% Increase Over Pretreatment
Control	6	57.23	57.26	-
Natural bGH 25 mg/day	5	58.54	65.50	11.9
pRec 2/3 bGH 25 mg/day	6	57.48	63.34	10.2

Each cow was injected daily subcutaneously with either placebo or bGH solution once daily for 10 days.

Claims

Claims for the following Contracting States: BE, CH, DE, FR, GB, IT, LI, NL, SE

1. A plasmid for the production of an animal growth hormone or polypeptide analog thereof having substantially the same amino acid sequence as, and the biological activity of, the naturally-occurring hormone in a suitable Escherichia coli host cell containing the thermolabile repressor C_I which plasmid renders the Escherichia coli host cell capable, upon increasing the temperature of the host cell to a temperature at which the repressor is inactivated, of effecting expression of DNA encoding the hormone

or polypeptide analog thereof, wherein the plasmid comprises:

a double-stranded DNA molecule which comprises in 5' to 3' order the following:

the promoter and operator P_LO_L from λ bacteriophage;

an N utilization site for binding antiterminator N protein produced by the host cell;

a mutant C_{II} ribosomal binding site having the sequence

TAAGGAAGTACTTACAT

ATTCCTTCATGAATGTA;

an ATG initiation codon;

a DNA sequence encoding the hormone or polypeptide analog to be expressed in phase with the ATG initiation codon;

and which additionally includes a DNA sequence which contains an origin of replication from the bacterial plasmid pBR322 capable of autonomous replication in the Escherichia coli host cell and a gene associated with a selectable or identifiable phenotypic trait which is manifested when the plasmid is present in the Escherichia coli host cell.

2. A plasmid of claim 1, wherein the Escherichia coli host cell is strain A1637.

3. A plasmid of claim 1, wherein the plasmid is circular.

4. A plasmid of claim 1, wherein the temperature is above 42° C.

5. A plasmid of claim 1, wherein the phenotypic trait is drug resistance or temperature sensitivity.

6. A plasmid of claim 5, wherein the drug resistance is resistance to ampicillin, chloramphenicol or tetracycline.

7. A plasmid of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes bovine growth hormone

8. A plasmid of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes human growth hormone.

9. A plasmid of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes porcine growth hormone

10. A plasmid of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes chicken growth hormone

11. A plasmid for production of bovine growth hormone of claim 7 designated pRec 2/3 having the restriction map shown in Fig 2 and deposited under ATCC Accession No. 39385.

12. A plasmid for production of bovine growth hormone of claim 7 designated pRoll having the restriction map shown in Fig. 3 and deposited under ATCC Accession No. 39390.

13. A plasmid for production of human growth hormone of claim 8 designated pTV18(1) having the restriction map shown in Fig 4 and deposited under ATCC Accession No. 39386.

14. A plasmid for production of human growth hormone of claim 8 designated pTV104(2) having the

restriction map shown in Fig. 4 and deposited under ATCC Accession No. 39384.

15. A host plasmid system for production of an animal growth hormone or polypeptide analog thereof comprising the plasmid of claim 1 in a suitable host.

16. A host plasmid system of claim 15, wherein the host is Escherichia coli strain A1637.

17. A host plasmid system for production of bovine growth hormone comprising the plasmid of claim 7 in a suitable host.

18. A host plasmid system for production of bovine growth hormone comprising the plasmid of claim 11 in a suitable host.

19. A host plasmid system for production of bovine growth hormone comprising the plasmid of claim 12 in a suitable host.

20. A host plasmid system for production of human growth hormone which comprises the plasmid of claim 13 in a suitable host.

21. A host plasmid system for production of human growth hormone comprising the plasmid of claim 14 in a suitable host.

22. A method for producing an animal growth hormone or polypeptide analog thereof which comprises growing the host plasmid system of claim 15 under suitable conditions permitting production of the growth hormone or analog and recovering the resulting growth hormone or polypeptide analog thereof.

23. A method of claim 22, wherein the suitable conditions comprise growth of the host plasmid system for an appropriate period of time at 42° C followed by continued growth at 37-39° C for an additional period of time, said growth being carried out in a suitable medium.

24. A method of claim 23, wherein the appropriate period of time at 42° C is 10 to 30 minutes and the additional period of time at 37-39° C is sufficient to make the total period of growing time 60 minutes to 90 minutes.

25. A method of claim 24, wherein the appropriate period of time at 42° C is about 15 minutes and the additional period of time is about 75 minutes at 38-39° C.

26. A method of claim 23, wherein the suitable medium is lactalbumin hydrolysate with addition of glucose or brain heart infusion.

27. A method for producing bovine growth hormone which comprises growing the host plasmid system of claims 18 under suitable conditions permitting production of bGH and recovering the resulting bGH.

28. A method for producing bovine growth hormone which comprises growing the host plasmid system of claim 19 under suitable conditions permitting production of bGH and recovering the resulting bGH.

29. A method for producing human growth hormone which comprises growing the host plasmid system of claims 20 under suitable conditions permitting production of hGH and recovering the resulting human growth hormone.

30. A method for producing human growth hormone which comprises growing the host plasmid system of claim 21 under suitable conditions permitting production of hGH and recovering the resulting human growth hormone.

Claims for the following Contracting State: AT

1. A method for producing an animal growth hormone or polypeptide analog thereof having substantially the same amino acid sequence as, and the biological activity of, the corresponding naturally-occurring

hormone which comprises growing a suitable Escherichia coli host cell containing the thermolabile repressor C_I and a plasmid capable of expressing the hormone or polypeptide analog thereof in the host cell, under suitable conditions permitting production of the hormone or polypeptide analog thereof and recovering the resulting hormone or polypeptide analog thereof, the plasmid comprising a double-stranded DNA molecule which includes in 5' to 3' order the following:

the promoter and operator P_LO_L from λ bacteriophage;

an N utilization site for binding antiterminator N protein produced by the host cell;

a mutant C_{II} ribosomal binding site having the sequence

TAAGGAAGTACTTACAT

ATTCCTTCATGAATGTA;

an ATG initiation codon;

a DNA sequence encoding the hormone or polypeptide analog to be expressed in phase with the ATG initiation codon;

and which additionally includes a DNA sequence which contains an origin of replication from the bacterial plasmid pBR322 capable of autonomous replication in the Escherichia coli host cell and a gene associated with a selectable or identifiable phenotypic trait which is manifested when the plasmid is present in the Escherichia coli host cell.

2. A method of claim 1, wherein the Escherichia coli host cell is strain A1637.

3. A method of claim 1, wherein the plasmid is circular.

4. A method of claim 1, wherein the temperature is above 42° C.

5. A method of claim 1, wherein the phenotypic trait is drug resistance or temperature sensitivity.

6. A method of claim 5, wherein the drug resistance is resistance to ampicillin, chloramphenicol or tetracycline.

7. A method of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes bovine growth hormone.

8. A method of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes porcine growth hormone.

9. A method of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes chicken growth hormone

10. A method of claim 1, wherein the DNA encoding the hormone or polypeptide analog encodes human growth hormone

11. A method of claims 1 and 7, wherein there is used a plasmid being designated pRec 2/3 having the restriction map shown in Fig. 2 and being deposited under ATCC Accession No. 39385.

12. A method of claims 1 and 7, wherein there is used a plasmid being designated pRoll having the restriction map shown in Fig. 3 and being deposited under ATCC Accession No. 39390.

13. A method of claims 1 and 10, wherein there is used a plasmid being designated pTV18(1) having the restriction map shown in Fig. 4 and being deposited under ATCC Accession No. 39386.

14. A method of claims 1 and 10, wherein there is used a plasmid being designated pTV104(2) having the restriction map shown in Fig. 4 and being deposited under ATCC Accession No. 39384.

15. A method of claim 1, wherein the host plasmid system is grown for an appropriate period of time at 42°C and thereafter at 37-39°C for an additional period of time, the growth being carried out in a suitable medium.

16. A method of claim 15, wherein the appropriate period of time at 42°C is 10 to 30 minutes and the additional period of time at 37-39°C is sufficient to make the total period of growing time 60 minutes to 90 minutes.

17. A method of claim 16, wherein the appropriate period of time at 42°C is about 15 minutes and the additional period of time is about 75 minutes at 38-39°C.

18. A method of claim 15, wherein the suitable medium is lactalbumin hydrolysate with addition of glucose or brain heart infusion.

Revendications

Revendications pour les Etats contractants suivants: BE CH DE FR GB IT LI NL SE

1. Plasmide pour la production d'une hormone de croissance animale ou d'un analogue polypeptidique de celle-ci, comportant pratiquement la même séquence d'aminoacides et la même activité biologique que l'hormone existant dans la nature, dans une cellule hôte appropriée de Escherichia coli contenant le répresseur thermolabile C_I, lequel plasmide rend la cellule hôte de Escherichia coli capable, lorsque la température de la cellule hôte est élevée jusqu'à une température à laquelle le répresseur est inactivé, d'effectuer l'expression de l'ADN codant pour l'hormone ou l'analogue polypeptidique de celle-ci, ledit plasmide comprenant:
une molécule d'ADN bicaténaire qui comprend, dans l'ordre 5' à 3', les éléments suivants:
le promoteur et l'opérateur P_LO_L provenant du bactériophage λ;
un site d'utilisation de N pour la liaison de l'antiterminateur protéine N produit par la cellule hôte;
un site de liaison ribosomique C_{II} mutant comportant la séquence

TAAGGAAGTACTTACAT
ATTCCTTCATGAATGTA;

un codon d'initiation ATG;
une séquence d'ADN codant pour l'hormone ou l'analogue polypeptidique à exprimer en phase avec le codon d'initiation ATG;
et qui comprend en outre une séquence d'ADN qui contient une origine de réplication provenant du plasmide bactérien pBR322 capable de réplication autonome dans la cellule hôte de Escherichia coli et un gène associé à un caractère phénotypique sélectionnable ou identifiable qui est manifesté lorsque le plasmide est présent dans la cellule hôte de Escherichia coli.

2. Plasmide selon la revendication 1, pour lequel la cellule hôte de Escherichia coli est la souche A1637.

3. Plasmide selon la revendication 1, pour lequel le plasmide est circulaire.

4. Plasmide selon la revendication 1, pour lequel la température est supérieure à 42°C.

5. Plasmide selon la revendication 1, pour lequel le caractère phénotypique est la résistance à un médicament ou la sensibilité à la température.

6. Plasmide selon la revendication 5, pour lequel la résistance à un médicament est la résistance à l'ampicilline, au chloramphénicol ou à la tétracycline.

7. Plasmide selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidi-

que code pour l'hormone de croissance bovine.

8. Plasmide selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance humaine.

9. Plasmide selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance porcine.

10. Plasmide selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance du poulet.

11. Plasmide pour la production de l'hormone de croissance bovine selon la revendication 7, désigné par pRec 2/3, ayant la carte de restriction représentée sur la figure 2 et déposé sous le n° ATCC 39385.

12. Plasmide pour la production de l'hormone de croissance bovine selon la revendication 7, désigné par pROII, ayant la carte de restriction représentée sur la figure 3 et déposé sous le n° ATCC 39390.

13. Plasmide pour la production de l'hormone de croissance humaine selon la revendication 8, désigné par pTV18(1), ayant la carte de restriction représentée sur la figure 4 et déposé sous le n° ATCC 39386.

14. Plasmide pour la production de l'hormone de croissance humaine selon la revendication 8, désigné par pTV104(2), ayant la carte de restriction représentée sur la figure 4 et déposé sous le n° ATCC 39384.

15. Système hôte-plasmide pour la production d'une hormone de croissance animale ou d'un analogue polypeptidique de celle-ci, comprenant le plasmide de la revendication 1 dans un hôte approprié.

16. Système hôte-plasmide selon la revendication 15, dans lequel l'hôte est la souche A1637 de Escherichia coli.

17. Système plasmide-hôte pour la production de l'hormone de croissance bovine, comprenant le plasmide de la revendication 7 dans un hôte approprié.

18. Système plasmide-hôte pour la production de l'hormone de croissance bovine, comprenant le plasmide de la revendication 11 dans un hôte approprié.

19. Système plasmide-hôte pour la production de l'hormone de croissance bovine, comprenant le plasmide de la revendication 12 dans un hôte approprié.

20. Système plasmide-hôte pour la production de l'hormone de croissance humaine, comprenant le plasmide de la revendication 13 dans un hôte approprié.

21. Système plasmide-hôte pour la production de l'hormone de croissance humaine, comprenant le plasmide de la revendication 14 dans un hôte approprié.

22. Procédé pour la production d'une hormone de croissance animale ou d'un analogue polypeptidique de celle-ci, comprenant la culture du système hôte-plasmide de la revendication 15, dans des conditions appropriées permettant la production de l'hormone de croissance ou de l'analogue, et la récupération de hormone de croissance résultante ou de son analogue polypeptidique résultant.

23. Procédé selon la revendication 22, dans lequel les conditions appropriées comprennent la culture du système hôte-plasmide à 42° C pendant une durée convenable, suivie d'une culture poursuivie à 37-39° C pendant une durée supplémentaire, ladite culture étant effectuée dans un milieu approprié.

24. Procédé selon la revendication 23, dans lequel la durée convenable à 42° C est de 10 à 30 minutes et la durée supplémentaire à 37-39° C est suffisante pour porter à 60-90 minutes la durée totale de la culture.

25. Procédé selon la revendication 24, dans lequel la durée convenable à 42° C est d'environ 15 minutes

et la durée supplémentaire est d'environ 75 minutes à 38-39 °C.

26. Procédé selon la revendication 23, dans lequel le milieu approprié est l'hydrolysat de lactalbumine avec addition de glucose ou le bouillon cerveau coeur.

27. Procédé pour la production de l'hormone de croissance bovine (HCb), comprenant la culture du système hôteplasmide de la revendication 18 dans des conditions appropriées permettant la production d'HCb, et la récupération de l'HCb résultante.

28. Procédé pour la production de l'hormone de croissance bovine, comprenant la culture du système hôte-plasmide de la revendication 19 dans des conditions appropriées permettant la production d'HCb, et la récupération de l'HCb résultante.

29. Procédé pour la production de l'hormone de croissance humaine (HCh), comprenant la culture du système hôteplasmide de la revendication 20 dans des conditions appropriées permettant la production d'HCh, et la récupération de l'hormone de croissance humaine résultante.

30. Procédé pour la production de l'hormone de croissance humaine, comprenant la culture du système hôte-plasmide de la revendication 21 dans des conditions appropriées permettant la production d'HCh, et la récupération de l'hormone de croissance humaine résultante.

Revendications pour l'Etat contractant suivant: AT

1. Procédé pour la production d'une hormone de croissance animale ou d'un analogue polypeptidique de celle-ci, comportant pratiquement la même séquence d'aminoacides et la même activité biologique que l'hormone correspondante existant dans la nature, lequel comprend la culture d'une cellule hôte appropriée de Escherichia coli contenant le répresseur thermolabile C_I et un plasmide capable d'exprimer l'hormone ou son analogue polypeptidique dans la cellule hôte, dans des conditions appropriées permettant la production de l'hormone ou de son analogue polypeptidique, et la récupération de l'hormone résultante ou de son analogue polypeptidique résultant, le plasmide comprenant une molécule d'ADN bicaténaire qui comprend, dans l'ordre 5' à 3', les éléments suivants:
   le promoteur et l'opérateur P_LO_L provenant du bactériophage λ;
   un site d'utilisation de N pour la liaison de l'antiterminateur protéine N produit par la cellule hôte;
   un site de liaison ribosomique C_{II} mutant comportant la séquence

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TAAGGAAGTACTTACAT
ATTCCTTCATGAATGTA;
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   un codon d'initiation ATG;
   une séquence d'ADN codant pour l'hormone ou l'analogue polypeptidique à exprimer en phase avec le codon d'initiation ATG;
   et qui comprend en outre une séquence d'ADN qui contient une origine de réplication provenant du plasmide bactérien pBR322 capable de réplication autonome dans la cellule hôte de Escherichia coli et un gène associé à un caractère phénotypique sélectionnable ou identifiable qui est manifesté lorsque le plasmide est présent dans la cellule hôte de Escherichia coli.

2. Procédé selon la revendication 1, dans lequel la cellule hôte de Escherichia coli est la souche A1637.

3. Procédé selon la revendication 1, dans lequel le plasmide est circulaire.

4. Procédé selon la revendication 1, dans lequel la température est supérieure à 42 °C.

5. Procédé selon la revendication 1, dans lequel le caractère phénotypique est la résistance à un médicament ou la sensibilité à la température.

6. Procédé selon la revendication 5, dans lequel la résistance à un médicament est la résistance à

l'ampicilline, au chloramphénicol ou à la tétracycline.

7. Procédé selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance bovine.

8. Procédé selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance porcine.

9. Procédé selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance du poulet.

10. Procédé selon la revendication 1, dans lequel l'ADN codant pour l'hormone ou l'analogue polypeptidique code pour l'hormone de croissance humaine.

11. Procédé selon les revendications 1 et 7, dans lequel on utilise un plasmide désigné par pRec 2/3, ayant la carte de restriction représentée sur la figure 2 et déposé sous le n° ATCC 39385.

12. Procédé selon les revendications 1 et 7, dans lequel on utilise un plasmide désigné par pROII, ayant la carte de restriction représentée sur la figure 3 et déposé sous le n° ATCC 39390.

13. Procédé selon les revendications 1 et 10, dans lequel on utilise un plasmide désigné par pTV18(1), ayant la carte de restriction représentée sur la figure 4 et déposé sous le n° ATCC 39386.

14. Procédé selon les revendications 1 et 10, dans lequel on utilise un plasmide désigné par pTV104(2), ayant la carte de restriction représentée sur la figure 4 et déposé sous le n° ATCC 39384.

15. Procédé selon la revendication 1, dans lequel le système hôte-plasmide est cultivé à 42°C pendant une durée convenable et ensuite à 37-39°C pendant une durée supplémentaire, ladite culture étant effectuée dans un milieu approprié.

16. Procédé selon la revendication 15, dans lequel la durée convenable à 42°C est de 10 à 30 minutes et la durée supplémentaire à 37-39°C est suffisante pour porter à 60-90 minutes la durée totale de la culture.

17. Procédé selon la revendication 16, dans lequel la durée convenable à 42°C est d'environ 15 minutes et la durée supplémentaire est d'environ 75 minutes à 38-39°C.

18. Procédé selon la revendication 15, dans lequel le milieu approprié est l'hydrolysat de lactalbumine avec addition de glucose ou le bouillon cerveau coeur.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten: BE, CH, DE, FR, GB, IT, LI, NL, SE

1. Plasmid zur Herstellung eines tierischen Wachstumshormons oder einem Polypeptidanalogon davon, mit im wesentlichen derselben Aminosäuresequenz und derselben biologischen Aktivität des natürlichen Hormons in einer geeigneten Escherichia coli-Wirtzelle, die den thermolabilen Repressor C_I enthält,

wobei das Plasmid die Escherichia coli-Wirtzelle bei einer Erhöhung der Wirtzellentemperatur auf eine Temperatur, bei der der Repressor inaktiviert wird, befähigt, das Hormon oder dessen Polypeptidanalogon codierende DNS zu exprimieren,

wobei das Plasmid ein doppelsträngiges DNS-Molekül umfaßt, das in 5' nach 3' Richtung

- den Promotor und Operator P_LO_L eines λ-Bakteriophagen;
- eine N-Erkennungsstelle zum Binden von durch die Wirtzelle gebildetem Antiterminator-N-Protein;
- eine Mutante C_{II} ribosomale Bindungsstelle der Sequenz:

TAAGGAAGTACTTACAT
ATTCCTTCATGAATGTA;

- ein ATG-Initiationscodon;
- eine DNS-Sequenz, die das in Phase mit dem ATG-Initiationscodon zu exprimierende Hormon oder Polypeptidanalogon codiert,

aufweist und

das darüberhinaus eine DNS-Sequenz umfaßt, die einen Replikationsursprung aus dem bakteriellen Plasmid pBR322 enthält, und die zur autonomen Replikation in der Escherichia coli-Wirtzelle fähig ist und ein Gen umfaßt, das mit einer selektierbaren oder identifizierbaren phänotypischen Eigenschaft einhergeht, die dann auftritt, wenn das Plasmid in der Escherichia coli-Wirtzelle vorhanden ist.

2. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß es sich bei der Escherichia coli-Wirtzelle um den Stamm A1637 handelt.

3. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß es ringförmig ist.

4. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß die Temperatur oberhalb 42 °C liegt.

5. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß das Phänotypergebnis aus einer Arzneimittelresistenz oder Temperaturempfindlichkeit besteht.

6. Plasmid nach Anspruch 5, dadurch gekennzeichnet, daß die Arzneimittelresistenz aus einer Ampicillin-, Chloramphenicol- oder Tetracyclinresistenz besteht.

7. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge Rinderwachstumshormon codiert.

8. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge menschliches Wachstumshormon codiert.

9. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge Schweinewachstumshormon codiert.

10. Plasmid nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge Hühnerwachstumshormon codiert.

11. Plasmid zur Gewinnung von Rinderwachstumshormon nach Anspruch 7, das als pRec 2/3 bezeichnet ist, die in Fig. 2 dargestellte Restriktionskarte aufweist und unter der ATCC-Hinterlegungsnummer 39385 hinterlegt ist.

12. Plasmid zur Gewinnung von Rinderwachstumshormon nach Anspruch 7, das als pROII bezeichnet ist, die in Fig. 3 dargestellte Restriktionskarte aufweist und unter der ATCC-Hinterlegungsnummer 39390 hinterlegt ist.

13. Plasmid zur Gewinnung von menschlichem Wachstumshormon nach Anspruch 8, das als pTV18(1) bezeichnet ist, die in Fig. 4 dargestellte Restriktionskarte aufweist und unter der ATCC-Hinterlegungsnummer 39386 hinterlegt ist.

14. Plasmid zur Gewinnung von menschlichem Wachstumshormon nach Anspruch 8, das als pTV104(2) bezeichnet ist, die in Fig. 4 dargestellte Restriktionskarte aufweist und unter der ATCC-Hinterlegungsnummer 39384 hinterlegt ist.

15. Wirtplasmidsystem zur Gewinnung eines menschlichen Wachstumshormons oder eines Polypeptidanalogen desselben, umfassend das Plasmid gemäß Anspruch 1 in einem geeigneten Wirt.

16. Wirtplasmidsystem nach Anspruch 15, dadurch gekennzeichnet, daß der Wirt aus dem Escherichia coli-Stamm A1637 besteht.

17. Wirtplasmidsystem zur Gewinnung von Rinderwachstumshormon, umfassend das Plasmid nach Anspruch 7 in einem geeigneten Wirt.

18. Wirtplasmidsystem zur Gewinnung eines Rinderwachstumshormons, umfassend das Plasmid nach Anspruch 11 in einem geeigneten Wirt.

19. Wirtplasmidsystem zur Gewinnung eines Rinderwachstumshormons, umfassend das Plasmid nach Anspruch 12 in einem geeigneten Wirt.

20. Wirtplasmidsystem zur Gewinnung von menschlichem Wachstumshormon, umfassend das Plasmid nach Anspruch 13 in einem geeigneten Wirt.

21. Wirtplasmidsystem zur Gewinnung eines menschlichen Wachstumshormons, umfassend das Plasmid nach Anspruch 14 in einem geeigneten Wirt.

22. Verfahren zur Gewinnung eines tierischen Wachstumshormons oder Polypeptidanalogen desselben durch Wachsenlassen des Wirtplasmidsystems nach Anspruch 15 unter geeigneten Bedingungen, die die Bildung des Wachstumshormons oder Analogen und Gewinnung des gebildeten Wachstumshormons oder Polypeptidanalogen desselben gestatten.

23. Verfahren nach Anspruch 22, dadurch gekennzeichnet, daß man unter geeigneten Bedingungen arbeitet, indem man das Wirtplasmidsystem eine geeignete Zeit lang bei 42 °C wachsen und dann zusätzlich bei einer Temperatur von 37 - 39 °C weiterwachsen läßt, wobei das Wachsen auf einem geeigneten Medium erfolgt.

24. Verfahren nach Anspruch 23, dadurch gekennzeichnet, daß das Wachsenlassen 10 - 30 min lang bei 42 °C und so lange bei 37 - 39 °C erfolgt, bis die Gesamtwachstumsdauer 60 - 90 min beträgt.

25. Verfahren nach Anspruch 24, dadurch gekennzeichnet, daß das Wachsenlassen etwa 15 min lang bei 42 °C und etwa 75 min lang bei 38 - 39 °C erfolgt.

26. Verfahren nach Anspruch 23, dadurch gekennzeichnet, daß man als geeignetes Wachstumsmedium ein mit Glucose oder Gehirn/Herz-Aufguß(infusion) versetztes Lactalbuminhydrolysat verwendet.

27. Verfahren zur Gewinnung von Rinderwachstumshormon, dadurch gekennzeichnet, daß man das Wirtplasmidsystem nach Anspruch 18 unter die Bildung von bGH gestattenden geeigneten Bedingungen wachsen läßt und das gebildete bGH abtrennt.

28. Verfahren zur Gewinnung von Rinderwachstumshormon, dadurch gekennzeichnet, daß man das Wirtplasmidsystem gemäß Anspruch 19 unter die Bildung von bGH gestattenden geeigneten Bedingungen wachsen läßt und das gebildete bGH abtrennt.

29. Verfahren zur Gewinnung von menschlichem Wachstumshormon, dadurch gekennzeichnet, daß man das Wirtplasmidsystem gemäß Anspruch 20 unter die Bildung von hGH gestattenden geeigneten Bedingungen wachsen läßt und das gebildete hGH abgetrennt.

30. Verfahren zur Gewinnung von menschlichem Wachstumshormon, dadurch gekennzeichnet, daß man das Wirtplasmidsystem gemäß Anspruch 21 unter die Bildung von hGH gestattenden geeigneten Bedingungen wachsen läßt und das gebildete menschliche Wachstumshormon abtrennt.

Patentansprüche für folgenden Vertragsstaaten: AT

1. Verfahren zur Herstellung eines tierischen Wachstumshormons oder eines Polypeptidanalogons davon mit im wesentlichen derselben Aminosäuresequenz und derselben biologischen Aktivität des korrespondierenden natürlich vorkommenden Hormons, umfassend das Vermehren einer geeigneten Esche-

richia coli-Wirtzelle, die den thermolabilen Repressor C_I und ein Plasmid enthält, das unter geeigneten Bedingungen zur Expression des Hormons oder des Peptidanalogons davon in der Wirtzelle fähig ist, die die Bildung des Hormons oder des Polypeptidanalogons davon erlauben und das Gewinnen des resultierenden Hormons oder des Polypeptidanalogons davon, wobei das Plasmid ein doppelsträngiges DNA-Molekül umfaßt, das in 5' nach 3' Richtung,

- den Promotor und Operator P_LO_L eines λ-Bakteriophagen;
- eine N-Erkennungsstelle zum Binden von durch die Wirtzelle gebildetem Antiterminator-N-Protein;
- eine Mutante C_{II} ribosomale Bindungsstelle der Sequenz

TAAGGAAGTACTTACAT

ATTCCTTCATGAATGTA;

- ein ATG-Initiationscodon;
- eine DNS-Sequenz, die das in Phase mit dem ATG-Initiationscodon zu exprimierende Hormon oder Polypeptidanalogon codiert,

aufweist und

das darüberhinaus eine DNS-Sequenz umfaßt, die einen Replikationsursprung aus dem bakteriellen Plasmid pBR322 enthält, und die zur autonomen Replikation in der Escherichia coli-Wirtzelle fähig ist und ein Gen umfaßt, das mit einer selektierbaren oder identifizierbaren phänotypischen Eigenschaft einhergeht, die dann auftritt, wenn das Plasmid in der Escherichia coli-Wirtzelle vorhanden ist.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß es sich bei der Escherichia coli-Wirtzelle um den Stamm A1637 handelt.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Plasmid ringförmig ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Temperatur oberhalb 42 °C liegt.

5. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Phänotypergebnis aus einer Arzneimittelresistenz oder Temperaturempfindlichkeit besteht.

6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß die Arzneimittelresistenz aus einer Ampicillin-, Chloramphenicol- oder Tetracyclinresistenz besteht.

7. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge Rinderwachstumshormon codiert.

8. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge Schweinewachstumshormon codiert.

9. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge Hühnerwachstumshormon codiert.

10. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die DNS mit Codierung für das Hormon oder Polypeptidanaloge menschliches Wachstumshormon codiert.

11. Verfahren nach Ansprüchen 1 und 7, dadurch gekennzeichnet, daß man ein mit pRec 2/3 bezeichnetes, die in Figur 2 dargestellte Restriktionskarte aufweisendes und unter der ATCC-Hinterlegungsnummer 39385 hinterlegtes Plasmid verwendet.

12. Verfahren nach Ansprüchen 1 und 7, dadurch gekennzeichnet, daß man ein als pROII bezeichnetes, die in Figur 3 dargestellte Restriktionskarte aufweisendes und unter der ATCC-Hinterlegungsnummer 39390 hinterlegtes Plasmid verwendet.

13. Verfahren nach Ansprüchen 1 und 10, dadurch gekennzeichnet, daß man ein mit pTV18(1) bezeichne-

tes, die in Figur 4 dargestellte Restriktionskarte aufweisendes und unter der ATCC-Hinterlegungsnummer 39386 hinterlegtes Plasmid verwendet.

14. Verfahren nach Ansprüchen 1 und 10, dadurch gekennzeichnet, daß man ein als pTV104(2) bezeichnetes, die in Figur 4 dargestellte Restriktionskarte aufweisendes und unter der ATCC-Hinterlegungsnummer 39384 hinterlegtes Plasmid verwendet.

15. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man das Wirtplasmidsystem eine geeignete Zeit lang bei 42 °C wachsen und danach bei 37 - 39 °C weiterwachsen läßt, wobei das Wachstum in einem geeigneten Medium erfolgt.

16. Verfahren nach Anspruch 15, dadurch gekennzeichnet, daß man (das Wirtplasmidsystem) 10 - 30 min lang bei 42 °C und danach so lange bei 37 - 39 °C wachsen läßt, daß die gesamte Wachstumsdauer 60 - 90 min beträgt.

17. Verfahren nach Anspruch 16, dadurch gekennzeichnet, daß man (das Wirtplasmidsystem) etwa 15 min bei 42 °C und danach etwa 75 min bei 38 - 39 °C wachsen läßt.

18. Verfahren nach Anspruch 15, dadurch gekennzeichnet, daß man als geeignetes Medium ein mit Glucose oder Gehirn/Herz-Aufguß(infusion) versetztes Lactalbuminhydrolysat verwendet.

FIG.1.

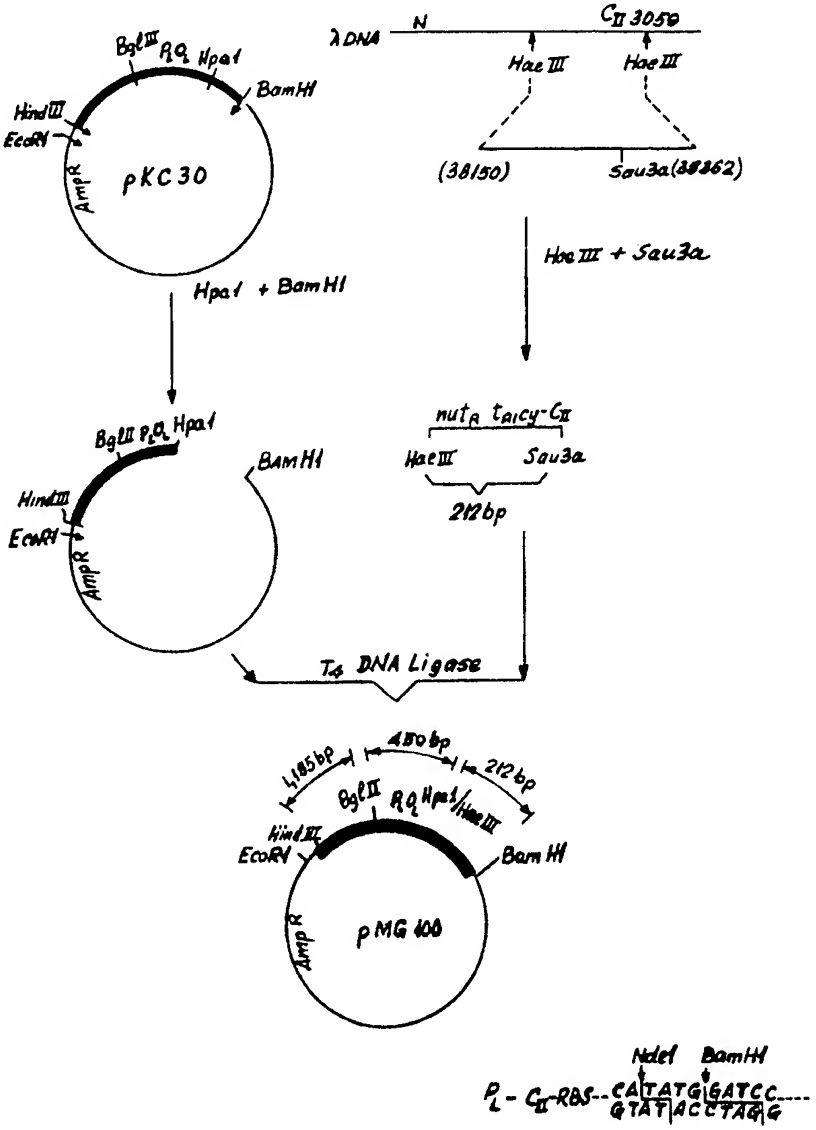

λ DNA
N
C II 3050
Hae III
Hae III
(38150)
Sau3a(38362)
Hae III + Sau3a
pKC30
BglII
PLPR
Hpa1
BamHI
HindIII
EcoRI
AmpR
Hpa1 + BamHI
nutR tRIcy-CII
Hae III
Sau3a
212bp
T4 DNA Ligase
485bp
450bp
212bp
BglII
PLPR
Hpa1/Hae III
HindIII
EcoRI
BamHI
AmpR
pMG100
NdeI
BamHI
PL - CII-RBS--CATATGGATCC----
GTATACCTAGG

FIG.2.

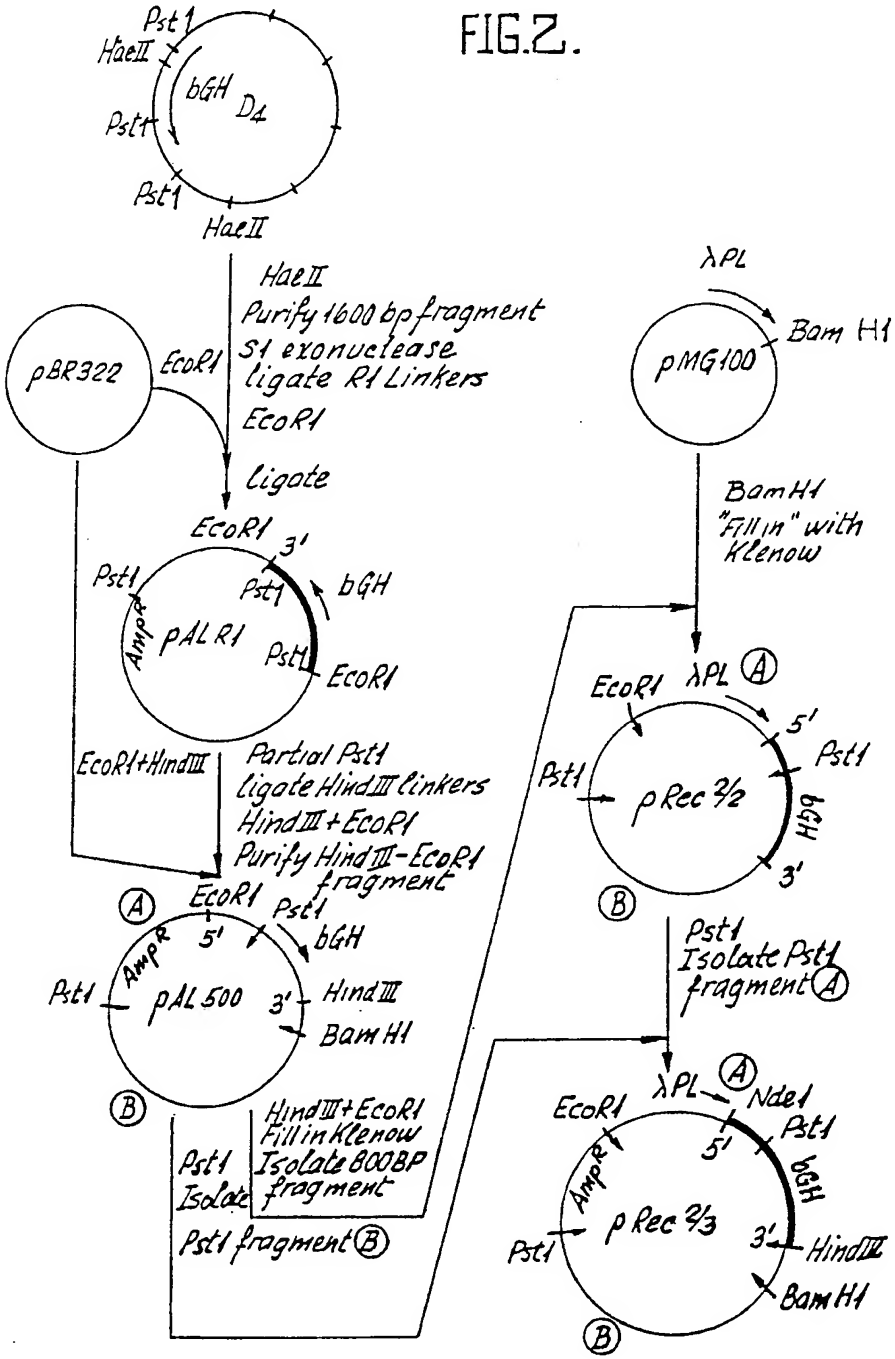

Pst1
HaeII
bGH
D4
Pst1
Pst1
HaeII
HaeII
Purify 1600 bp fragment
S1 exonuclease
ligate R1 Linkers
EcoR1
pBR322
EcoR1
Ligate
EcoR1
3'
Pst1
Pst1
bGH
Amp R
pAL R1
PstI
EcoR1
EcoR1+HindIII
Partial Pst1
ligate HindIII Linkers
HindIII + EcoR1
Purify HindIII-EcoR1 fragment
A
EcoR1
Pst1
bGH
AmpR
5'
Pst1
pAL 500
3'
HindIII
BamH1
B
HindIII+EcoR1
Fill in Klenow
Isolate 800BP fragment
Pst1
Isolate
Pst1 fragment B
λPL
pMG100
Bam H1
BamH1
"Fill in" with Klenow
EcoR1
λPL
A
5'
Pst1
Pst1
pRec 2/2
Hbg
3'
B
Pst1
Isolate Pst1 fragment A
λPL
A
Nde1
EcoR1
Pst1
5'
AmpR
bGH
Pst1
pRec 2/3
3'
HindIII
BamH1
B

FIG.3.

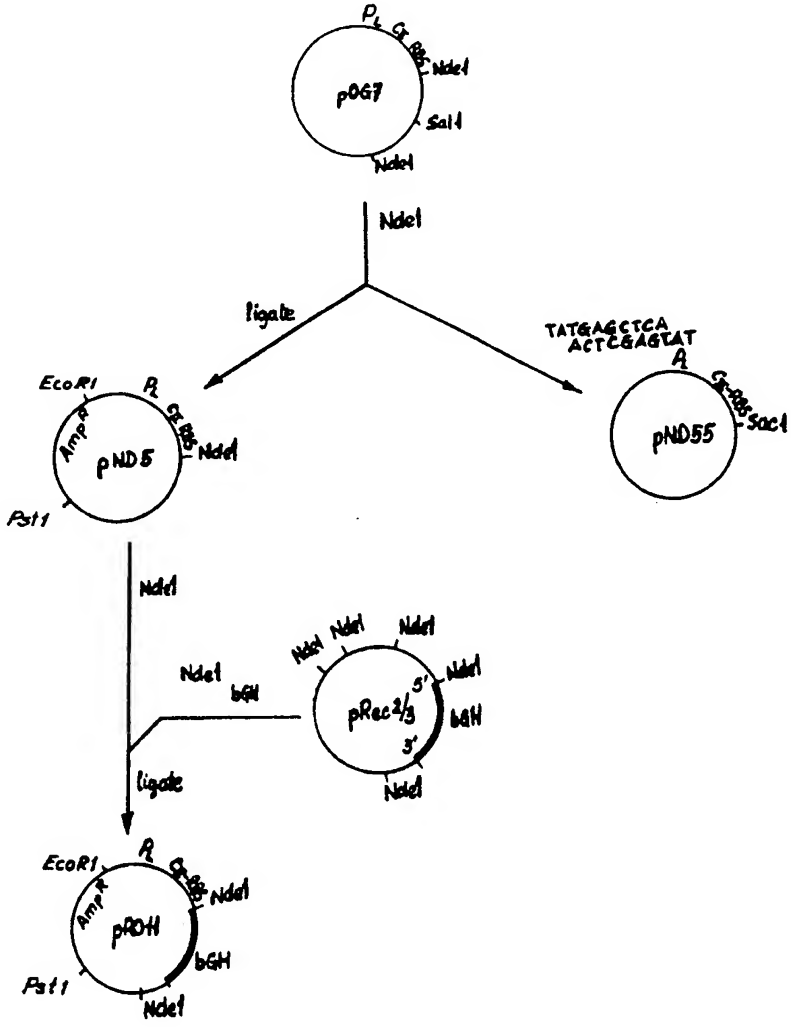

pOG7
NdeI
SalI
NdeI
NdeI
ligate
TATGAGCTCA
ACTCGAGTAT
A
pND55
SacI
EcoRI
Amp R
pND5
NdeI
PstI
NdeI
NdeI
NdeI
NdeI
NdeI
pRec2/3
5'
3'
bGH
NdeI
bGH
ligate
EcoRI
Amp R
pROH
NdeI
bGH
PstI
NdeI

FIG 4.

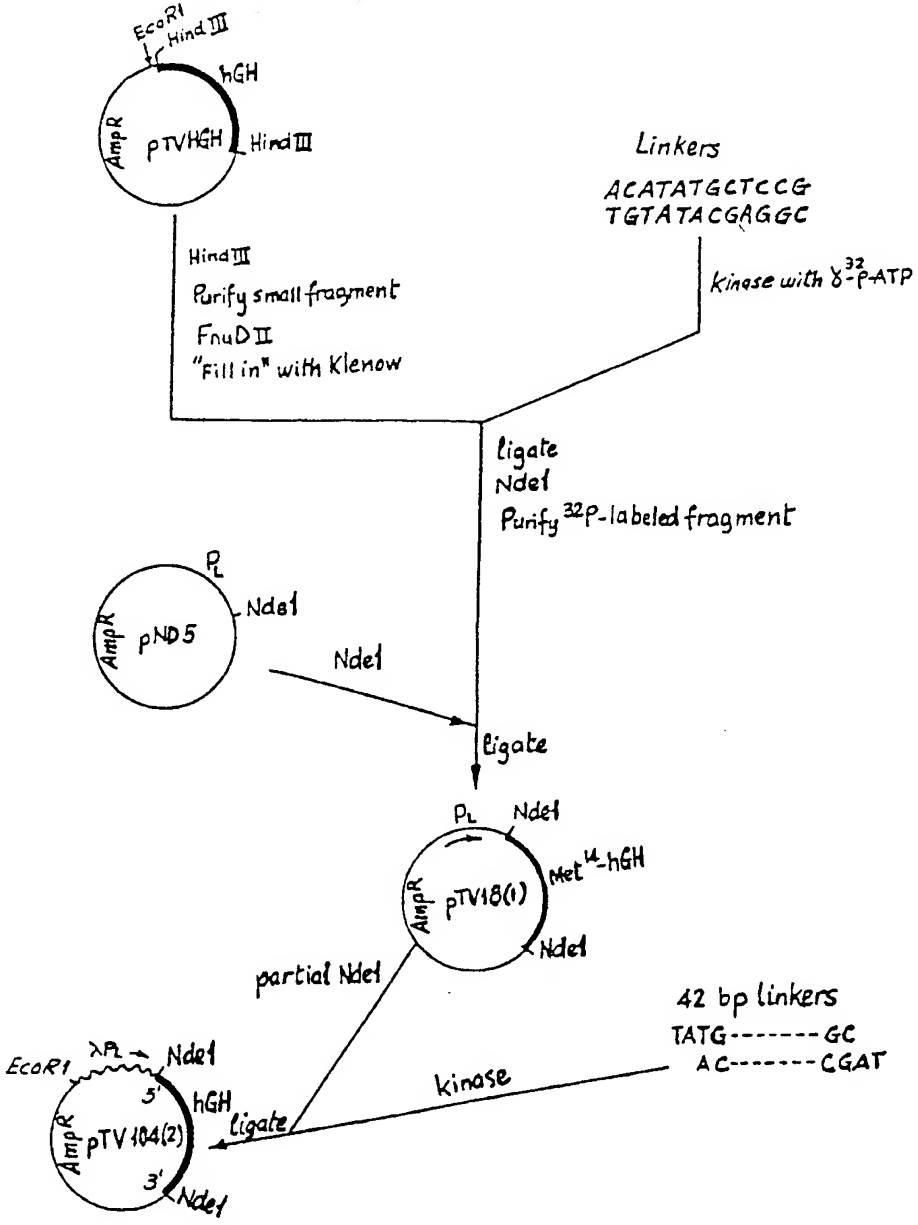

EcoR1
Hind III
hGH
AmpR
pTVHGH
Hind III
Linkers
ACATATGCTCCG
TGTATACGAGGC
Hind III
Purify small fragment
FnuD II
"Fill in" with Klenow
Kinase with γ-32P-ATP
ligate
Nde1
Purify 32P-labeled fragment
PL
Nde1
AmpR
pND5
Nde1
ligate
PL
Nde1
AmpR
pTV18(1)
Met14-hGH
Nde1
partial Nde1
42 bp linkers
TATG-------GC
AC-------CGAT
EcoR1
λPL
Nde1
5'
hGH
AmpR
pTV104(2)
3'
Nde1
ligate
Kinase

FIG.5.

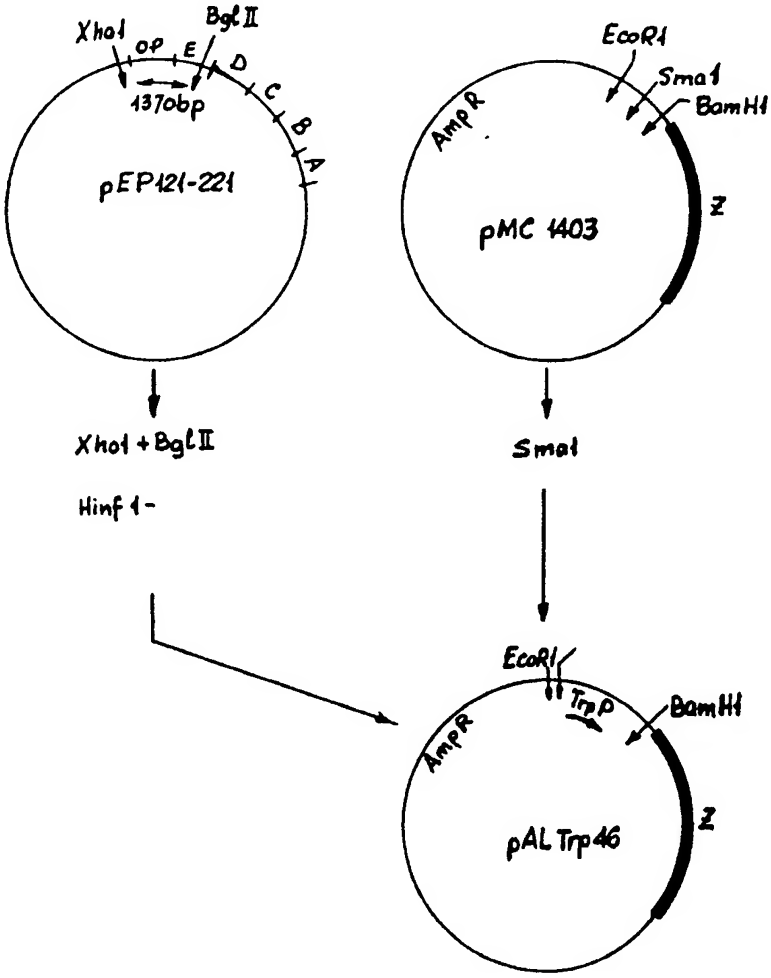

XhoI
BglII
OP
E
D
C
B
A
1370bp
pEP121-221
XhoI + BglII
Hinf 1-
EcoR1
SmaI
BamHI
AmpR
pMC 1403
Z
SmaI
EcoR1
TrpP
BamHI
AmpR
pAL Trp46
Z

FIG.6.

CONSTRUCTION OF Tac PROMOTER

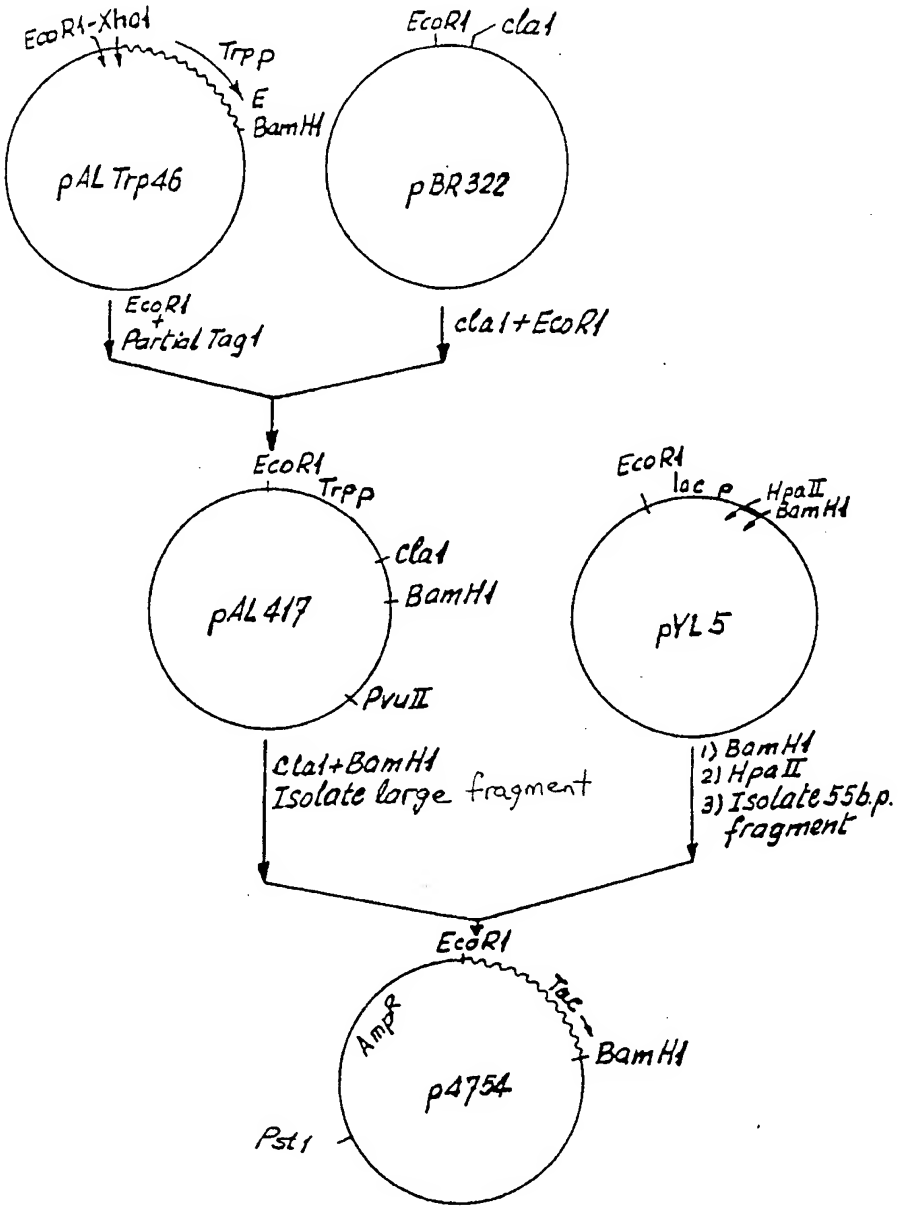
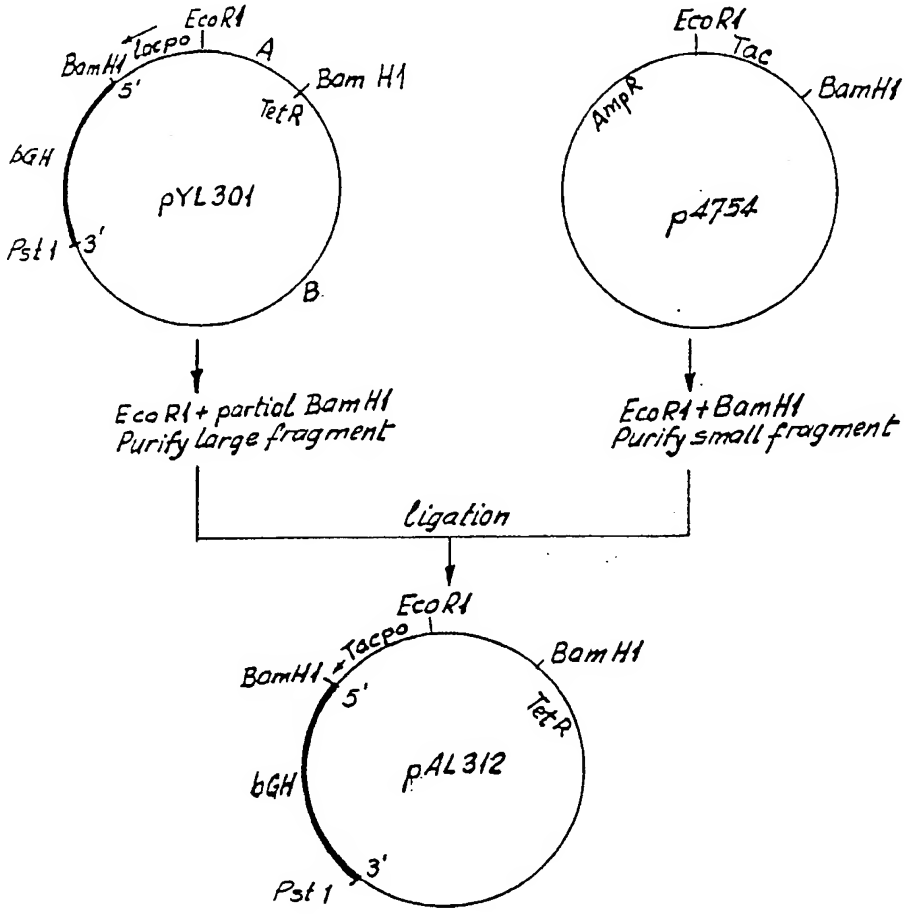


FIG.7.



Trp-35 region } TAC Promoter
Lac-40 region }

fMet Gly Ala Phe
---------CACACAGGAAACAG'GAT'CCT ATG GGC'GCC'TTC
S.D.

FIG.8.

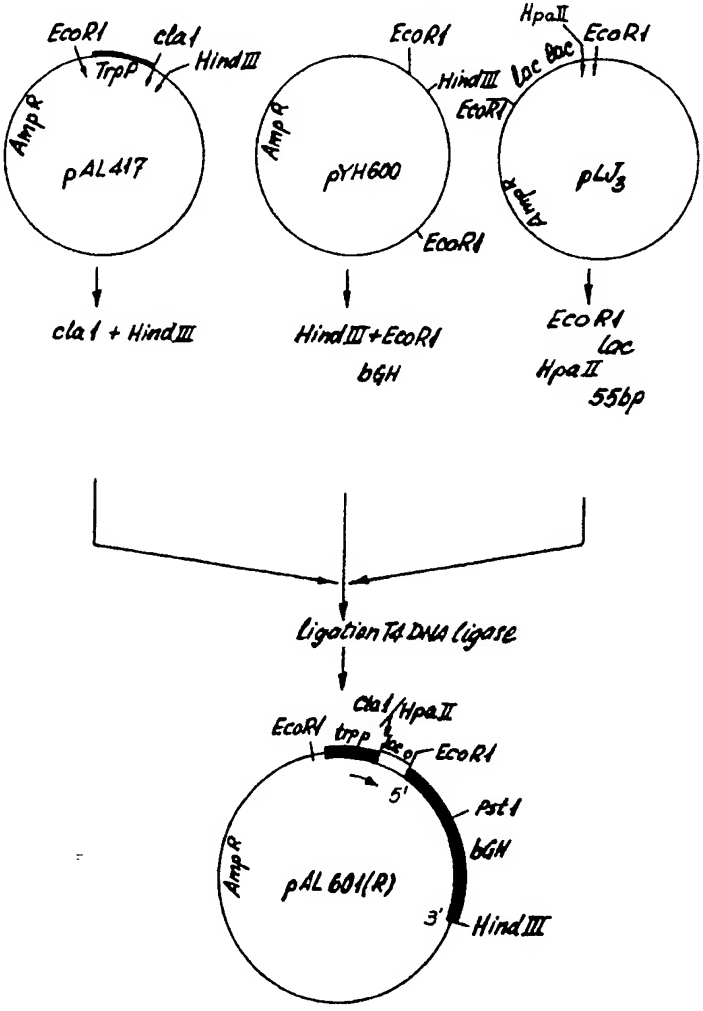

EcoR1
cla1
TrpP
HindIII
AmpR
pAL417
cla1 + HindIII
EcoR1
HindIII
AmpR
pYH600
EcoR1
HindIII+EcoR1
bGH
HpaII
EcoR1
Loc
EcoR1
AmpR
pLWJ3
EcoR1
Loc
HpaII
55bp
Ligation T4 DNA Ligase
Cla1/HpaII
EcoRV
trpp
Loc
EcoR1
5'
Pst1
bGH
AmpR
pAL 601(R)
3'
HindIII

FIG.9.

EXPRESSION OF bGH WITH His PROMOTER

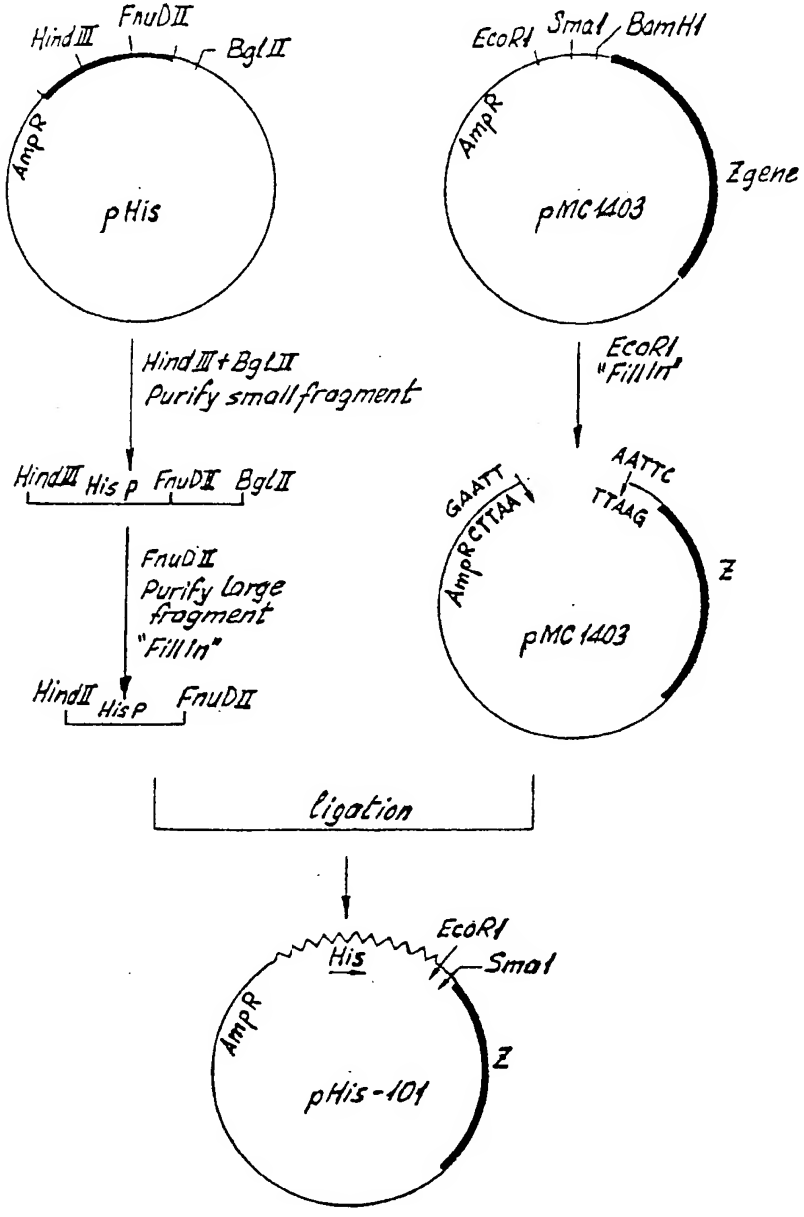


FIG.10.

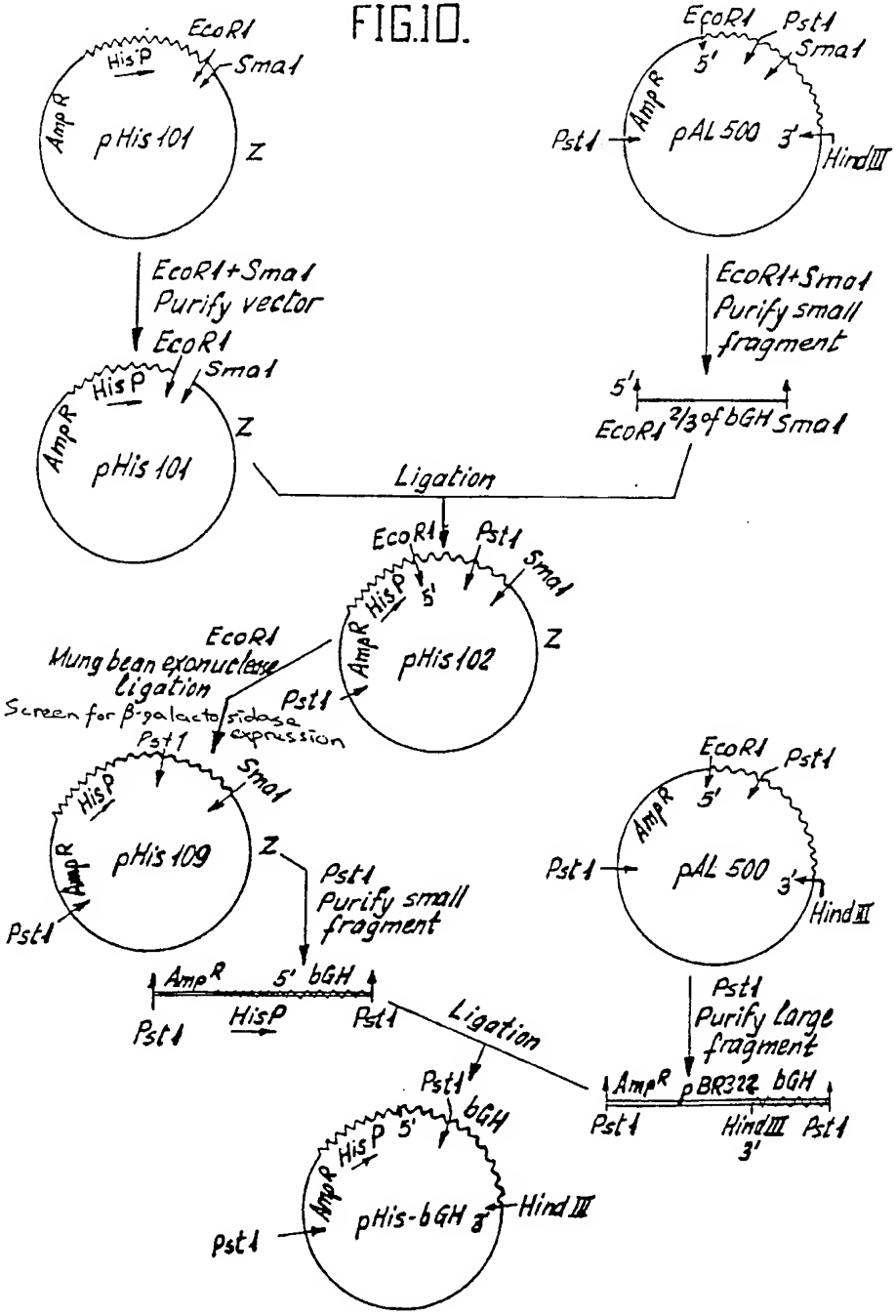

EcoR1
HisP
Sma1
Amp R
pHis 101
Z
Pst1
5'
3'
pAL 500
Hind III
EcoR1+Sma1
Purify vector
Purify small fragment
2/3 of bGH
Ligation
pHis 102
Mung bean exonuclease ligation
Screen for β-galactosidase expression
pHis 109
Pst1
Purify small fragment
Amp R
5' bGH
Purify large fragment
pBR322 bGH
bGH
pHis-bGH
3'

FIG11.

HAE II FRAGMENT WITH SYNTHETIC LINKER pYL-301

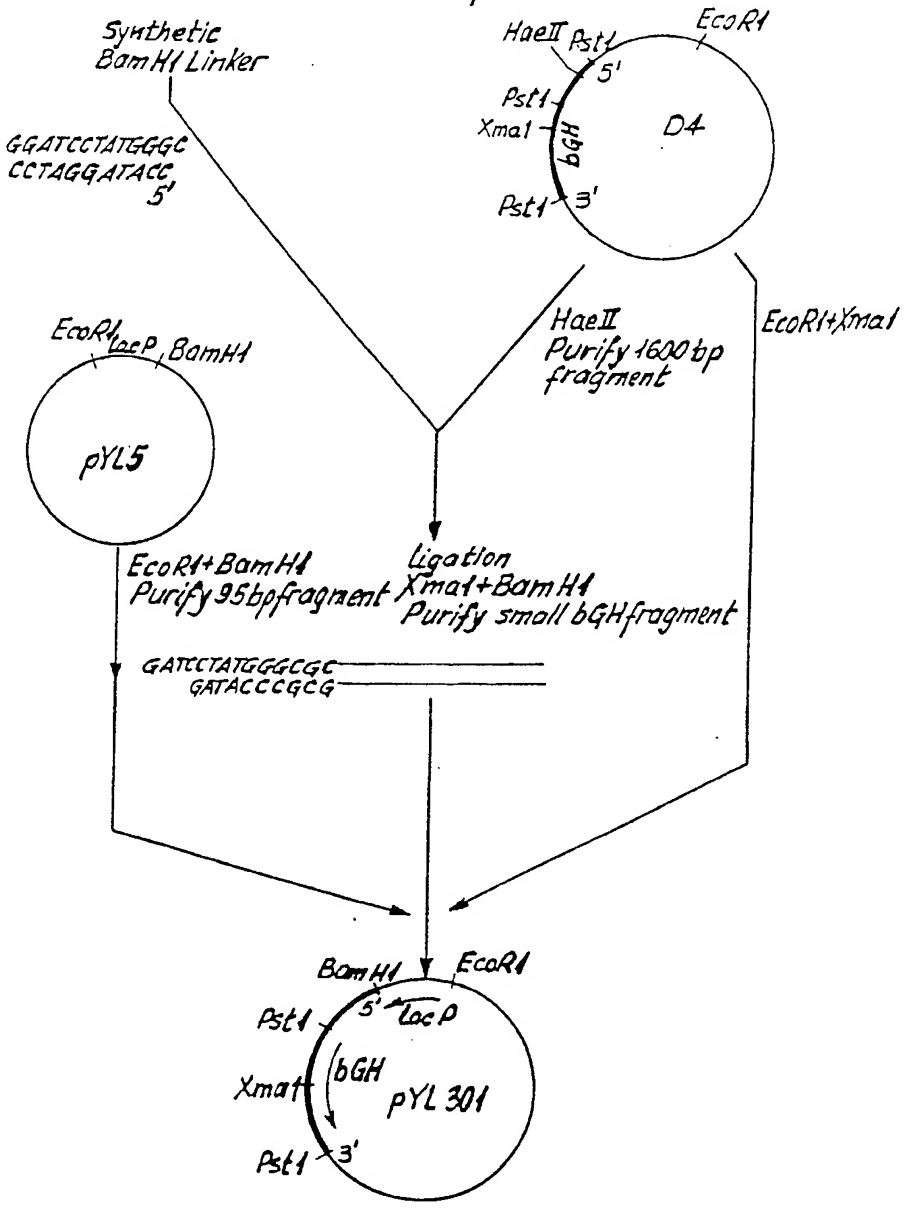


FIG.12.

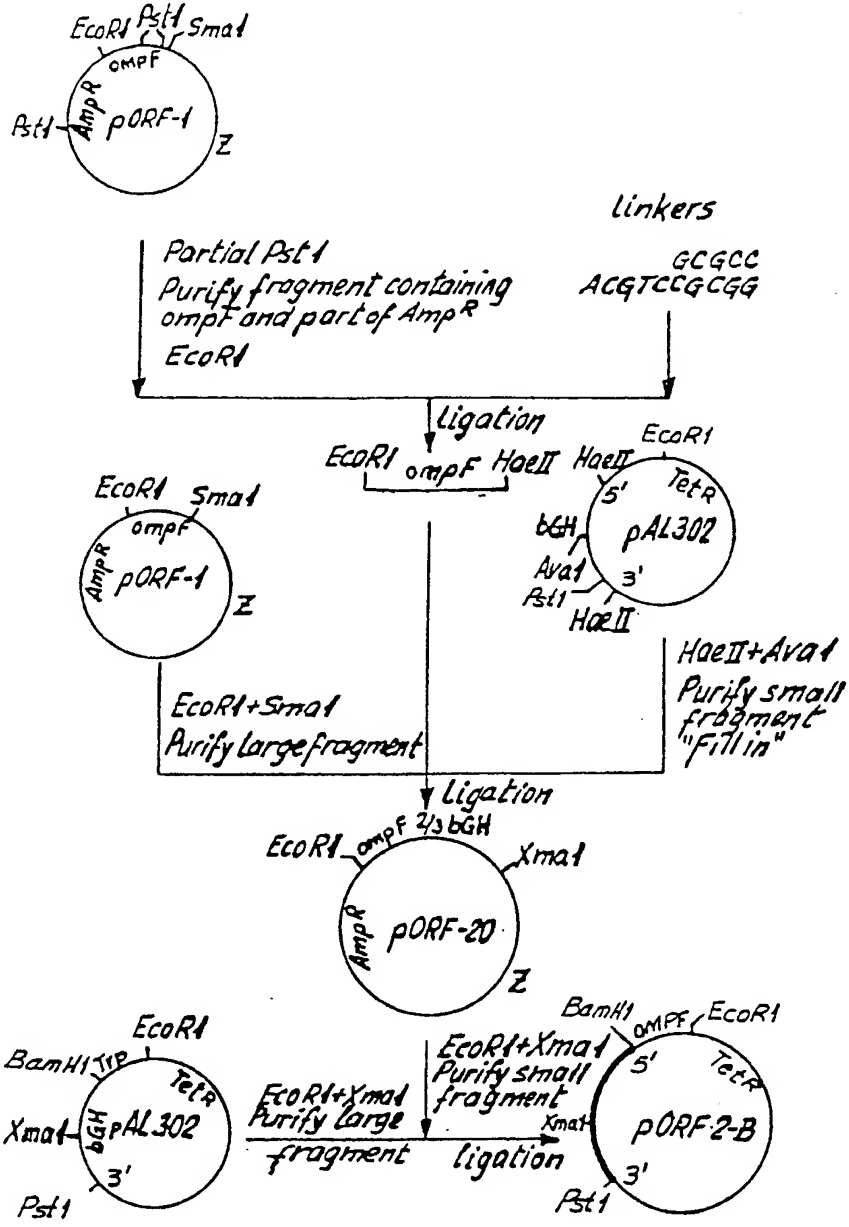

EcoR1
Pst1
Sma1
ompF
Amp^R
pORF-1
Z
linkers
GCGCC
ACGTCCGCGG
Partial Pst1
Purify fragment containing ompF and part of Amp^R
EcoR1
Ligation
EcoR1 ompF HaeII
HaeII
EcoR1
5'
Tet^R
bGH
pAL302
Ava1
3'
Pst1
HaeII
HaeII+Ava1
Purify small fragment "Fill in"
EcoR1+Sma1
Purify Large fragment
Ligation
EcoR1 ompF 2/3 bGH
Xma1
pORF-20
BamH1
TrP
EcoR1+Xma1
Purify small fragment
EcoR1+Xma1
Purify Large fragment
Xma1
ligation
pORF-2-B

FIG.13.

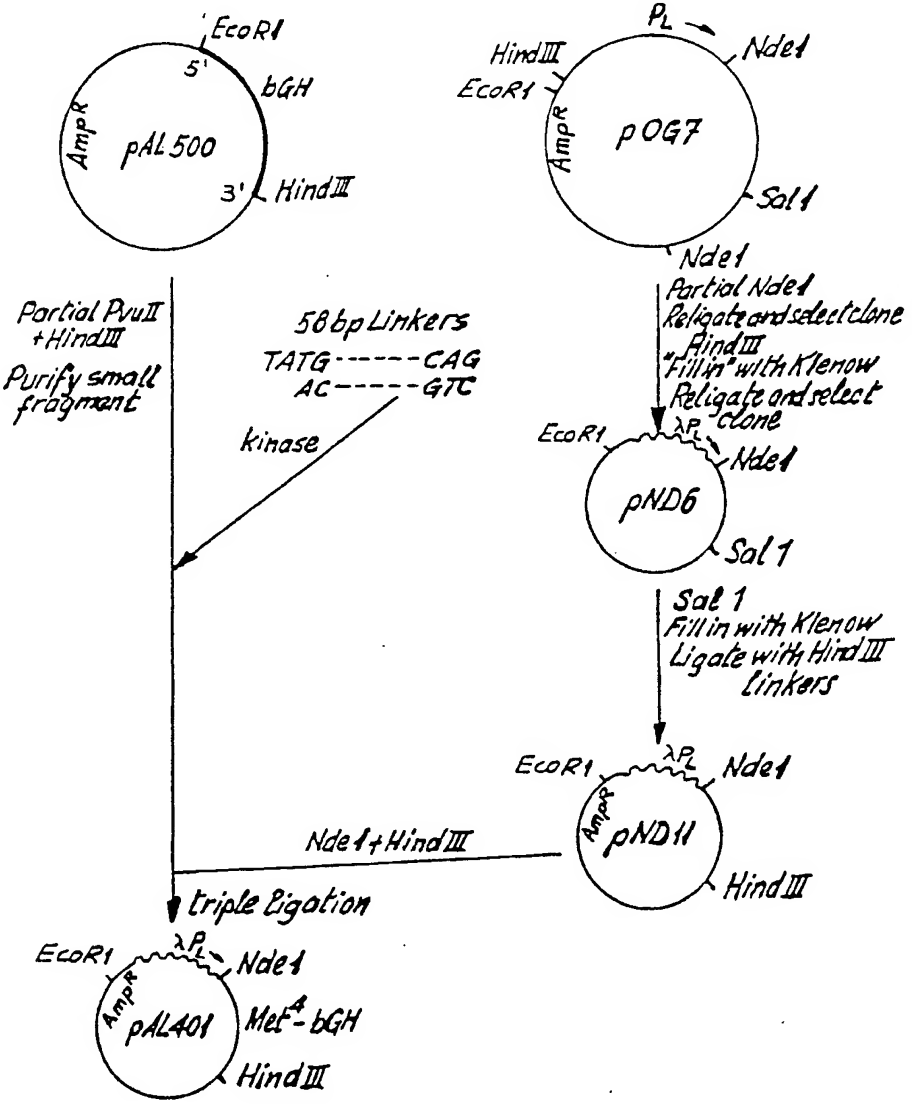

EcoR1
5'
bGH
AmpR
pAL500
3'
HindIII
PL
HindIII
Nde1
EcoR1
AmpR
pOG7
Sal1
Nde1
Partial Nde1
Religate and select clone
HindIII
"Fill in" with Klenow
Religate and select clone
Partial PvuII
+HindIII
Purify small fragment
58 bp Linkers
TATG------CAG
AC------GTC
kinase
EcoR1
λPL
Nde1
pND6
Sal1
Sal1
Fill in with Klenow
Ligate with HindIII
linkers
EcoR1
λPL
Nde1
AmpR
pND11
HindIII
Nde1+HindIII
triple ligation
EcoR1
λPL
Nde1
AmpR
pAL401
Met-4-bGH
HindIII

FIG.14.

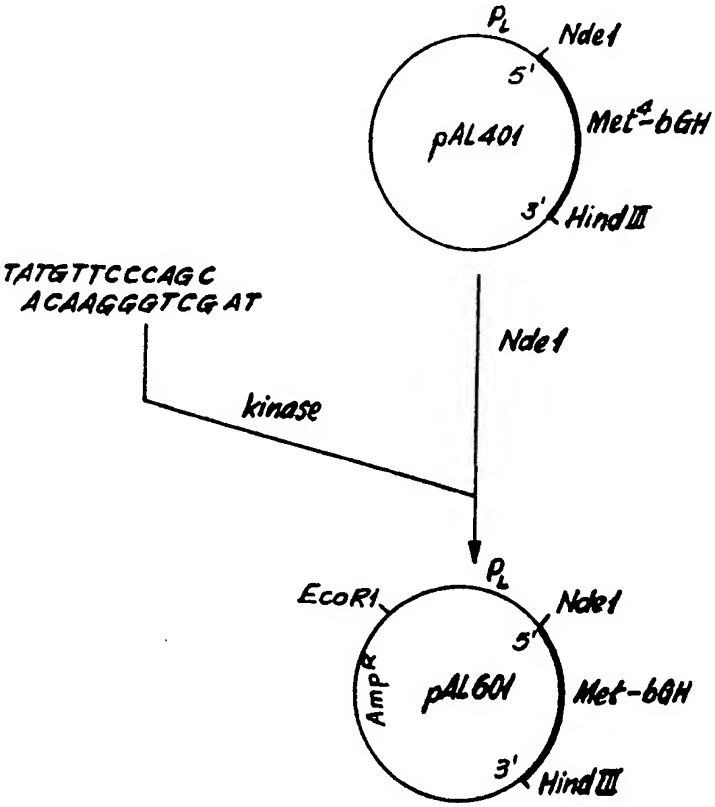

P_L
Nde1
5'
pAL401
Met^4-bGH
3'
Hind III
TATGTTCCCAGC
ACAAGGGTCG AT
kinase
Nde1
P_L
EcoR1
Nde1
5'
Amp^R
pAL601
Met-bGH
3'
Hind III

FIG.15.